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**Genetic regulation of neural crest cell differentiation**

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# **Genetic Regulation of Neural Crest Cell Differentiation**

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Biology and Biochemistry

August 2008

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# Abstract

Neural crest cells are a transient population of cells which differentiate into multiple derivatives. How these derivatives become specified is not well understood but Sox10 is known to be important in many of them. We are interested in defining the precise role of Sox10 in zebrafish melanophores. Current evidence suggests that the only vital function that Sox10 performs in melanophores is to induce expression of the melanocyte master regulator *mitfa* (Elworthy et al. 2003).

We explored a model for Sox10 function in melanophores, based upon a model for Sox10's role in mouse sympathetic neurons (Kim et al. 2003), and tested the following predictions: as well as inducing expression of *mitfa*, Sox10 will repress expression of genes downstream of Mitfa thus, Sox10 must be downregulated, via Mitfa, to allow melanophore differentiation. We observed derepression of melanophore marker genes in *sox10*<sup>3</sup> mutants, supporting the hypothesis that Sox10 represses these genes in wild type melanophores. We documented Sox10/*sox10* downregulation in developing melanophores and generated transgenic lines to test whether this is necessary for differentiation. Unfortunately our experimental lines did not express our transgene so we were unable to test this hypothesis. However, transgenic lines, generated as controls, which express CFP in melanophores or xanthophores will be useful tools in their own right. Finally we conducted RNA injection experiments to explore regulation of melanophore genes by Sox10 and Mitfa. We found that injection of *mitfa* induces expression of all our melanophore markers whereas co-injection of *mitfa* and *sox10* does not. We also found that the 7.2 kb *sox10* promoter contains six Mitf binding sites and is Mitfa responsive.

Our data broadly support our original model but also suggest that it does not describe the complete network. We propose a modified model for the role of Sox10 in the genetic regulatory network controlling melanophore development.

# Abbreviations

$\alpha$ -MSH	Anti Melanocyte Stimulating Hormone
$\alpha$ –DIG	Anti Dioxygenin
AMH	Anti Mullerian Hormone
ATR-16	Alpha Thalassemia Retardation
Bcl2	B-cell Leukaemia/Lymphoma 2
BH4	Tetrahydrobiopterin
bHLH-Zip	Basic Helix Loop Helix Leucine Zipper
BLAST	Basic Local Alignment Search Tool
BMP	Bone Morphogenic Protein
bp	base pairs
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
CBP	cAMP Binding Protein
ChIP	Chromatin Immunoprecipitation
Cl <sub>s</sub>	Colourless
CNS	Central Nervous System
Col2a1/11a2	Collagen Type 2 alpha 1/Type 11 alpha 2
CRE	cAMP Response Element
Dam	Deoxyadenosine Methylation
Dct	Dopachrome Tautomerase
DEPC	Diethylpyrocarbonate
df	Degrees of Freedom
DHICA	Dihydroxyindole-2-Carboxylic Acid
DIC	Differential Interference Contrast Microscopy
DMSO	Dimethyl Sulphoxide
DOM	Dominant Megacolon
dpf	Days Post Fertilisation
DRG	Dorsal Root Ganglia
E and P	Mouse staging, E, Days postcoitum or P, Days Post Birth
(E)CFP	(Enhanced) Cyan Fluorescent Protein
(E)GFP	(Enhanced) Green Fluorescent Protein
EDN3	Endothelin 3
EDNRB/1	Endothelin Receptor B/bl
EF-1 alpha	Elongation Factor 1 alpha
ES cells	Embryonic Stem cells
FGF	Fibroblast Growth Factor
Foxd3	Forkhead box D3
GAL4/UAS	Yeast Transcription Factor and its target sequence Upstream Activation Sequence
Gch	GTP Cyclohydrolase

GTP	Guanosine Triphosphate
HMG	High Mobility Group
HpF	Hours Post Fertilisation
Id3	Inhibitor of DNA Binding 3
ISH	In Situ Hybridisation
kb	kilo base pairs
LacZ	$\beta$ -Galactosidase
LB	Luria Broth
Lef1	Lymphocyte Enhancer Binding Factor 1
Ltk	Leucosine Tyrosine Kinase
MAG	Myelin Associated Glycoprotein
MAP kinase	Mitogen-Activated Protein Kinase
Mash1	Mammalian Achaete-Schute Homologue 1
Mitf/a	Microphthalmia
<i>Mi</i>	Mouse <i>Mitf</i> Mutant
MPZ	Myelin Protein Zero (P0)
Msx	Muscle Segment Homeobox
<i>Nac</i>	<i>nacre</i> , Zebrafish <i>mitfa</i> Mutant
NBT/BCIP	Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3'- Indoldyphosphate p-Toluidine Salt
NC	Neural Crest
Ngn1	Neurogenin 1
OCA1	Oculocutaneous Albinism Type 1A
OMIM	Online Mendelian Inheritance in Man
p300	E1A Binding Protein p300
PAGE	Polyacrylamide Gel Electrophoresis
Paics	Phosphoribosylaminoimidazole carboxylase
Pax3/7	Paired Box Transcription Factor 3/7
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
Phox2a/b	Paired-like Homeobox 2a/b
Pias3	Protein Inhibitor of Activated STAT 3
Pmel17	Pre-Melanosomal Protein 17
PRE	Pigmented Retinal Epithelium
PTU	1-Phenyl-2-thiourea
RET	'Rearranged during Transcription' proto-oncogene
RT-PCR	Reverse Transcription PCR
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium Dodecyl Sulphate
SE	Standard Error
Sox8/9/10	SRY-box containing gene 8/9/10
SRY	Sex Determining Region Y
SSC	Sodium Chloride Sodium Citrate
SUMO	Small Ubiquitin-like Modifier
SV40	Simian Virus 40
Tbx2	T-box Transcription Factor 2
Tcf	'Transcription Factor' family
TF	Transcription Factor
Tfe3/b/c	Transcription Factor Binding to IGHM Enhancer 3/b/c
TGF $\beta$	Transforming Growth Factor beta
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
TYR	Tyrosinase
Tyrp1/b/a	Tyrosinase-related Protein 1/1b/1a
WS1/2/3/4	Waardenburg Shah Syndrome 1/2/3/4
WT	Wild type
Xdh	Xanthine Dehydrogenase
XGal	5-Bromo-4-chloro-3-indoyl-B-D-Galactopyranoside

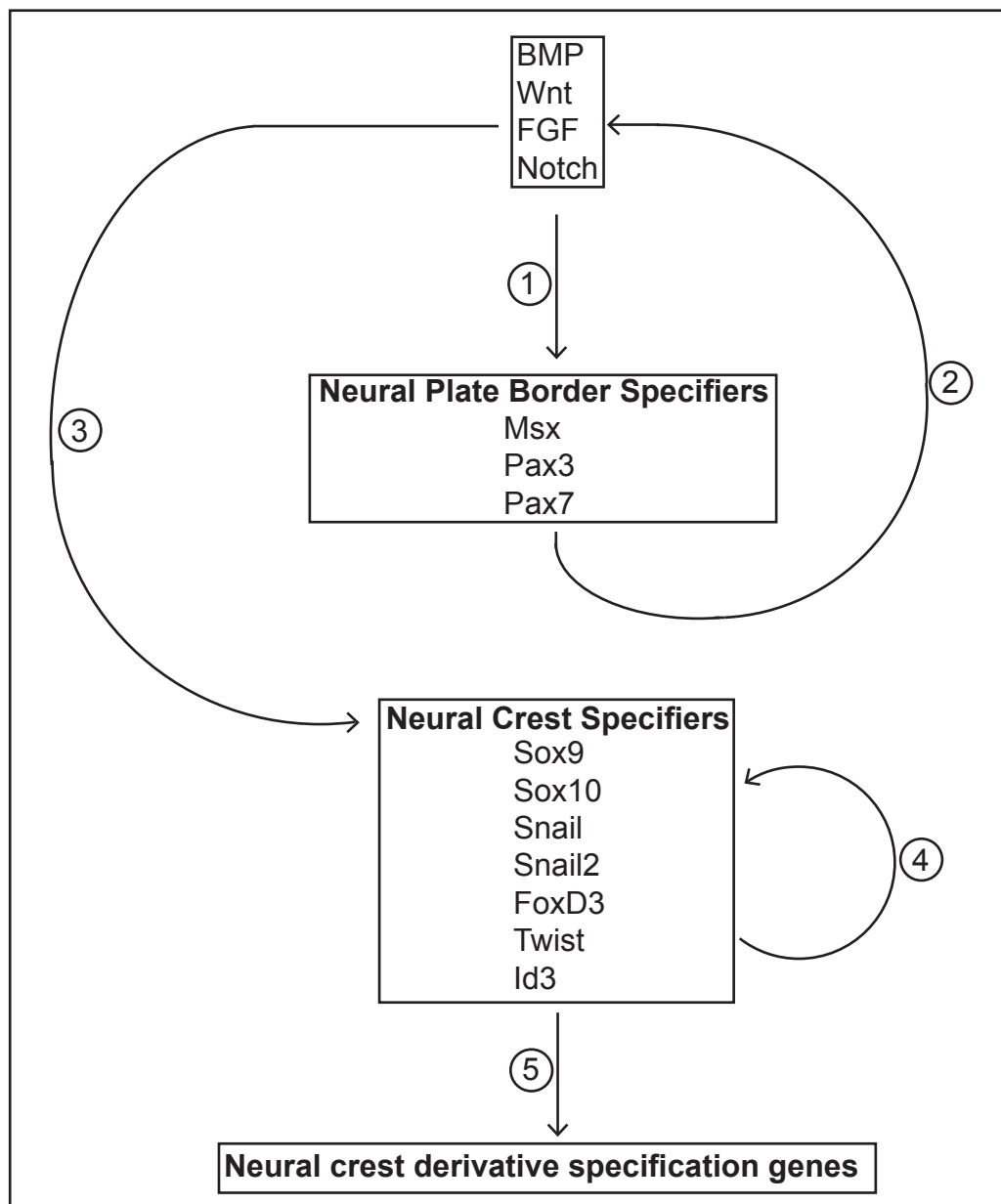
# Chapter 1

## Introduction

### 1.1 Induction and Specification of the Neural Crest

The neural crest is a group of cells which arise during neurulation at the junctions of neurectoderm and prospective epidermis and become the tips of the neural folds (Le Douarin and Kalcheim 1999). In fish, neurulation involves a thickening of the neurectoderm to form the neural keel and the most dorsolateral part of this becomes neural crest (Eisen and Weston 1993, Raible and Eisen 1994). Two major signaling pathways, BMP and Wnt, converge to induce neural crest (LaBonne and Bronner-Fraser 1998). BMP signaling at low levels appears to establish competency to become neural crest in cells at the edges of the neural plate; Wnt signals from adjacent ectoderm and underlying mesoderm then promote formation of neural crest (LaBonne and Bronner-Fraser 1998, Raible 2006, Nguyen et al. 1998). Other signaling pathways, FGF and Notch, also play a role in induction of Neural Plate Border Specifiers such as *Msx*, *Pax3* and *Pax7* (Meulemans and Bronner-Fraser 2004, Sauka-Spengler and Bronner-Fraser 2006, Sauka-Spengler and Bronner-Fraser 2008). In turn, these Neural Plate Border Specifiers modify the effects of BMP, FGF and Wnt signaling to induce expression of another set of genes termed Neural Crest Specifiers (Monsoro-Burq, Wang and Harland 2005, Steventon, Carmona-Fontaine and Mayor 2005). These genes include *Sox9* and *Sox10*, *Snail* and *Snail2*, *Foxd3*, *Twist* and *Id3*, the expression of which identifies neural crest. Neural Crest Specifiers are interdependent, regulating each other (Meulemans and Bronner-Fraser 2004, Sauka-Spengler and Bronner-Fraser 2008) as well as inducing expression of a wide variety of target genes which regulate specification and differentiation of the neural crest into its various derivatives (Figure 1.01).

Once specified as neural crest, these cells undergo an epidermal to mesenchymal transition and dissociate from the neural folds or neural keel. Neural crest cells migrate to many different parts of the developing embryo and differentiate into many



**Figure 1.01 Genes involved in the formation of neural crest**

A simplified diagram of the relationships between genes involved in neural crest formation. BMP, Wnt, FGF and Notch signalling induces expression of Neural Plate Border Specifiers (1). These genes modify the effects (2) of BMP, Wnt, FGF and Notch signalling on the expression of Neural Crest Specifiers (3). The expression of Neural Crest Specifiers identify cells as being neural crest. These genes are able to regulate each other (4) as well as induce expression of genes which specify neural crest derivatives (5).

different derivatives including neurons, glia, craniofacial cartilage and pigment cells. There are two pathways in the trunk that these cells migrate along, a lateral pathway between the epithelium and the somites and a medial pathway between the neural tube and the somites. In mouse, pigment cells migrate only on the lateral pathway and all other neural crest derivatives can be found on the medial pathway (Le Douarin and Kalcheim 1999). In zebrafish most neural crest derivatives, including some pigment cells, migrate on the medial pathway while only pigment cells migrate on the lateral pathway (Le Douarin and Kalcheim 1999, Raible et al. 1992, Kelsh 2004).

By in vitro primary culture of premigratory mouse, rat and *Xenopus* neural crest cells and by cell lineage tracing in zebrafish and chick, workers have demonstrated that neural crest cells are multipotent (Stemple and Anderson 1992, Stemple and Anderson 1993, Bronner-Fraser and Fraser 1988, Baroffio, Dupin and Le Douarin 1988, Collazo, Bronner-Fraser and Fraser 1993). Even migrating cells in chick have been shown to be multipotent in this way (Fraser and Bronner-Fraser 1991). Similar work has also shown that mammalian and avian neural crest cells can, in addition, be self renewing (Stemple and Anderson 1992, Baroffio, Dupin and Le Douarin 1991, Bronner-Fraser and Fraser, 1991). Within the embryo, multipotent neural crest cells become specified and then committed to specific fates and differentiate into the various neural crest derivatives. Fate specification has been defined as when a multipotent cell begins to display characteristics of a particular fate, e.g. a multipotent cell becomes a melanoblast when it begins to express melanocyte specific genes such as *Dct* or *Mitf* (Kelsh 2006). However, a cell which is specified may still ultimately differentiate as a different cell type. A cell becomes committed to a particular fate when it is unable to respond to signals that specify another fate. For example, a cell is committed as a melanoblast when it can no longer respond to glial specification signals, such as Notch signaling. Finally, differentiation describes the process between the time when a cell is fate specified and the time it becomes a fully specialized cell which displays all of the characteristics of a specific cell type. For example, a melanophore must express survival and migration genes such as *kit* as well as melanogenic enzymes such as Tyrosinase. However, it may be that cells are only committed to a specific fate under particular in vivo conditions and that this can be reversed if conditions change. If differentiated melanocytes are grown in specific culture conditions they can be encouraged to de-differentiate so that they express early crest marker genes; in doing so, a single melanocyte can then give rise to cells which are both multipotent and self-renewing (Real et al. 2006).

Whilst it is possible that neural crest-derived cells are directly fate specified from multipotent progenitors, evidence to date suggests that it is more likely that progenitors undergo progressive fate restriction. Here, multipotent cells become restricted to



form different precursors which have the potential to form an ever decreasing set of derivatives (Kelsh and Raible 2002, Henion and Weston 1997, Baroffio et al. 1991). For example, *sox10* mutants have defects in their non-skeletogenic derivatives (pigment cells, neurons, glia) but not in their skeletogenic derivatives (cartilage and fin mesenchyme) (Dutton et al. 2001, Southard-Smith, Kos and Pavan 1998, Herbarth et al. 1998), suggesting that the multipotent crest becomes restricted to precursors for each of those sub groups of crest cells (Dutton et al. 2001, Southard-Smith et al. 1998, Herbarth et al. 1998, Kelsh, Schmid and Eisen 2000). Neuronal and pigment lineages have also been demonstrated to segregate from one another during development (Henion and Weston 1997).

Exactly when specification occurs, particularly in relation to the timing of neural crest cell migration, is not clear and may vary between neural crest derivatives. In the case of pigment cells, labeling studies and observations of pigment cell markers in zebrafish suggest that these cell types may already be specified before migration and that cells have certainly become, or are becoming specified during migration (Raible and Eisen 1994, Lister et al. 1999, Kelsh et al. 2000, Parichy et al. 1999). Non-skeletogenic neural crest cells, including pigment cells, fail to specify in the zebrafish *sox10* mutant and most of these unspecified cells die in a premigratory position suggesting that specification occurs before, and may be necessary for, migration (Dutton et al. 2001). By transplantation of neural crest cells and differentiated melanocytes to different thoracic levels in chick embryos it has been shown that cells in this organism are required to be specified as melanocytes for them to be able to migrate along the dorso-lateral migration pathway (equivalent to lateral pathway in fish) (Erickson and Goins 1995). Indeed, specification of melanoblasts is delayed in Silkie chick embryos and cells do not begin to migrate in these embryos until they have become specified (Reedy, Faraco and Erickson 1998). However, some non-skeletogenic cells are able to migrate a short distance in zebrafish *sox10* mutants and these generally move to a DRG position (Dutton et al. 2001). The DRG contains progenitor cells which give rise to neurons later in development so even during wild type development these cells would be required to migrate without specifying. This suggests that specification is not necessary for migration per se but is important only in certain cell types or only in cells which migrate along the lateral pathway.

Many of the signals which are involved in neural crest induction are important again when individual neural crest derived cell types are specified. For example, Wnt signaling has been shown to be necessary early for neural crest induction but is also important later for specification of neural crest derived melanophores and cranial neural crest cells in the branchial arches (Lewis et al. 2004). Wnt signaling may also

play a role in the balance between pigment and neuronal cell types. However, different species appear to respond differently to increased Wnt signaling which promotes neuronal fates in mouse (Lee et al. 2004) and pigment fates in zebrafish (Dorsky, Moon and Raible 1998). Complex genetic interactions such as these make the study of the genetic regulation of neural crest cell differentiation an interesting field to work in. In particular, we are interested in the genetic interactions which regulate neural crest derived pigment cell differentiation.

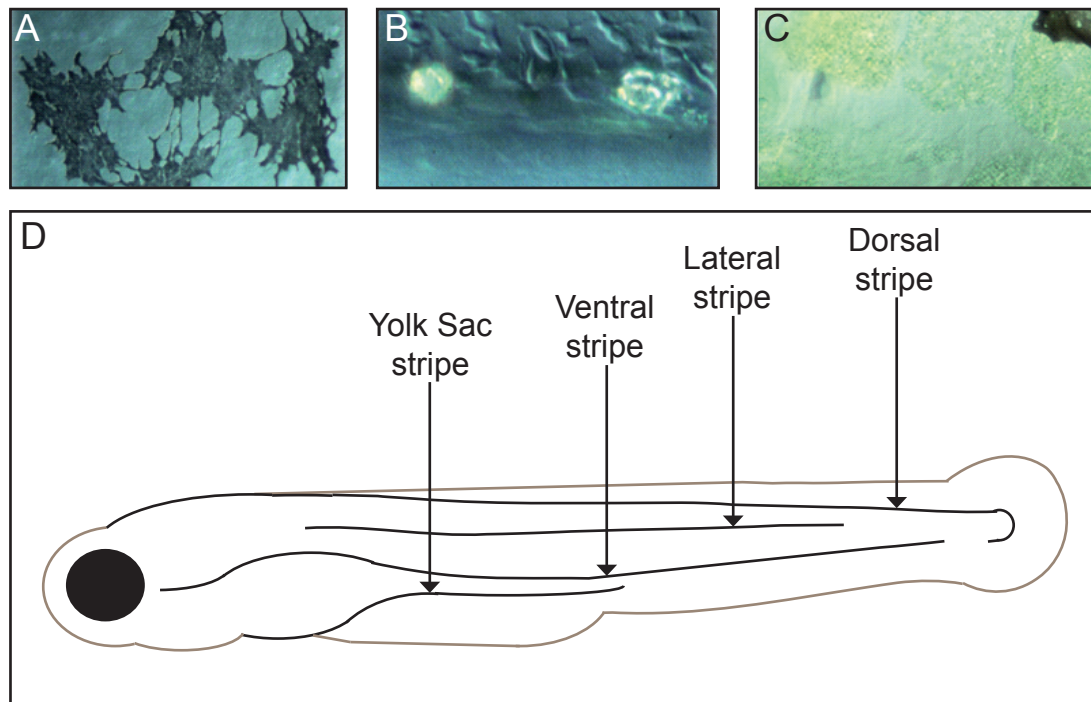
## 1.2 Pigment Cells

Whilst mammals and birds only have one pigment cell type arising from the neural crest, the melanocyte, zebrafish have three: black melanophores, yellow xanthophores and silver reflective iridophores (Lister et al. 1999). These cell types differ in their migration pathways; melanophores migrate on both the medial and the lateral pathway, iridophores migrate only along the medial pathway, and xanthophores migrate only on the lateral pathway (Le Douarin and Kalcheim 1999, Raible et al. 1992, Kelsh 2004). By 5 days post fertilisation (dpf) the pigment cells have reached their final destinations and their patterned distribution in the developing fish is clear. This consists of four stripes of melanophores one dorsally, one laterally, one ventrally and one under the yolk sac. All but the lateral stripe also contain iridophores. Xanthophores give a yellow cast to most of the embryo but are more concentrated dorsally (Figure 1.02).

### 1.2.1 Melanocyte development

Melanocytes make the pigment melanin, of which there are two kinds: black/brown eumelanin and red/yellow pheomelanin (Jackson et al. 1992, Zdarsky, Favor and Jackson 1990). Both of these are present in mice and humans, however zebrafish only have eumelanin. In melanocytes melanin is contained within organelles called melanosomes. In zebrafish these melanosomes remain within the melanophores but in mammals they are usually transported from melanocytes into keratinocytes to colour the skin/hair (Lin and Fisher 2007). This pigmentation provides both pattern to the skin/hair of individuals and important photoprotection which can diminish the risk of skin cancer (Kollias et al. 1991).

In zebrafish, melanoblasts can be identified in a premigratory neural crest position even before they pigment; from approximately 18 hpf they express melanoblast specific genes such as *mitfa* and *dct* (Kelsh et al. 2000, Lister et al. 1999). In mouse however melanoblasts can only be identified, by expression of *Dct*, once they are migrating at E10.5 (Steel, Davidson and Jackson 1992). Zebrafish melanoblasts migrate away from the premigratory position along both the medial and the lateral migration pathways



**Figure 1.02 Zebrafish pigmentation**

A-C Images taken from Kelsh (2004).

A. Melanophores are black and have a stellate morphology.

B. Iridophores are reflective and shine silvery under incident light.

C. Xanthophores have a yellow colouration.

D. Melanophores form four stripes in the zebrafish embryo the positions of which are illustrated in this diagram. Iridophores are also present in the dorsal, ventral and yolk sac stripes. Xanthophores give a yellow cast to most of the body but are more concentrated dorsally.

(Kelsh et al. 2000, Lister et al. 1999) and pigmentation occurs even whilst the cells are migrating from approximately 25 hpf. Mouse melanoblasts migrate along the lateral pathway only before they pass through the dermis to take up their final locations in the epidermis and hair follicles. It is only once they are at their final locations in the epidermis that mouse melanoblasts differentiate into melanocytes and pigment (Le Douarin and Kalcheim 1999). Hair follicle melanoblasts remain unpigmented and become melanocyte stem cells which then give rise to differentiated progeny (Lin and Fisher 2007).

We have already mentioned two genes expressed specifically in melanocytes, *Mitf* and *Dct*, and there are many others. Some of these genes play a role early in melanoblast development, to specify cells as melanoblasts and promote their differentiation; others encode enzymes necessary for melanin synthesis and processing. We will outline some of what is known about the melanocyte specific genes most relevant to our work here.

### **1.2.1i *Mitf***

*Mitf* is a basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factor. In mammals there are multiple isoforms of *Mitf* with different expression domains (reviewed Yasumoto et al. (1998)). We will be referring to the *Mitf-M* isoform which is expressed in the pigmented retinal epithelium (PRE) as well as in neural crest derived melanocytes (Nakayama et al. 1998, Lister et al. 1999). Zebrafish have two *mitf* genes which are expressed in the same cell types in zebrafish as *Mitf-M* is in mouse; *mitfa* is expressed in neural crest derived melanophores and the PRE and *mitfb* is only expressed in the PRE (Lister, Close and Raible 2001).

*Mitf* is used as a very early marker of melanocyte specification (reviewed Levy, Khaled and Fisher (2006), Goding (2000), Steingr msson, Copeland and Jenkins (2004)). It is described as the master regulator of melanocyte differentiation. This role is clear in mouse *Mitf* (*Mi*) homozygous mutants which lack all neural crest-derived melanocytes and have a loss, or severe reduction, in cells expressing melanophore markers (Opdecamp et al. 1997). Depending on the mutant allele, *Mi* mutants may also be deaf, have small eyes or reduced numbers of mast cells (Lister et al. 1999, Hodgkinson et al. 1993). Zebrafish *mitfa* (*nacre*) mutants have a similar phenotype to the *Mi* mutants showing a loss of melanophores as well as a decrease in xanthophores and a 40 % increase in iridophores (Lister et al. 1999). Mutations in *Mitf* in humans have comparable phenotypes to those of the mouse and zebrafish mutants resulting in Waardenburg syndrome (see below), Tietz syndrome (OMIM 103500) and a form of albinism (OMIM 103470).

Several Mitf overexpression studies have shown Mitf to be sufficient to cause cells to adopt a melanocyte fate, again demonstrating the importance of Mitf for melanocyte development. In chicken and quail neuroretinal cells, expression of Mitf induces rapid pigmentation of the cells (Planque et al. 1999) and injection of *mitfa* RNA into zebrafish embryos results in the formation of ectopic melanophores (Lister et al. 1999). Tachibana et al. (1996) showed that overexpression of Mitf in a fibroblast cell line causes the cells to take on a melanocyte morphology and express melanogenic genes such as *Dct*. However, this study has the caveat that the cell line used is already able to express Dct even before overexpression of Mitf. In a more recent paper, expression of Mitf in ES-like medaka cells produced what the authors reported to be fully differentiated melanocytes (Béjar, Hong and Scharl 2003). Overexpression of Mitf can cause melanocyte differentiation because Mitf is capable of regulating many genes characteristic of melanocytes. These include melanogenic enzymes (discussed in more detail later in this chapter) and survival factors such as c-Kit (Steingrímsson et al. 2004). However, other work implies that although it is necessary for Mitf to be expressed in a cell for it to adopt a melanocyte fate, Mitf expression alone is not sufficient for adoption of a melanocyte fate. Hou, Arnheiter and Pavan (2006) found that Sox10 is also required to be expressed in conjunction with Mitf for cells to express all of the melanocyte specific genes examined and fully differentiate as melanocytes. One report, from work carried out in a mouse melanoma cell line which already expresses melanogenic enzymes, shows that overexpression of Mitf in cell culture does not lead to increased expression of melanogenic enzymes (Gaggioli et al. 2003). The authors suggested that this shows that Mitf is not sufficient to induce expression of melanogenic genes. However, since the cells were already expressing these genes before the introduction of Mitf the work actually shows that Mitf is not sufficient to increase, rather than induce, expression of melanogenic genes. Cells from neural tube explants of *Kit<sup>W-LacZ</sup>* mice, which have reduced melanocyte survival, also have reduced levels of Mitf protein. Although this level of Mitf is sufficient to induce expression of melanogenic proteins Dct, Pmel17 and Tyrp1 it is not sufficient to induce expression of Tyrosinase, another melanogenic enzyme. This suggests that low levels of Mitf are not sufficient for complete melanocyte differentiation but is not informative about whether or not higher levels, perhaps above a threshold level, would be sufficient for cells to fully differentiate as melanocytes. c-Kit is also required for post-translational phosphorylation of Mitf (Hemesath et al. 1998) which both recruits the co-activator CBP/p300 to Mitf (Price et al. 1998) and marks Mitf for degradation (Wu et al. 2000). Thus, loss of c-Kit from these mice could result in reduced functionality of any Mitf protein that is present. This further complicates attempts to draw conclusions from the *Kit<sup>W-LacZ</sup>* mutant mouse about the necessity of Mitf for melanocyte differentiation.

Control of *Mitf* expression and thus of melanocyte specification is complicated, involving transcription factors such as Sox10, Pax3 and Lef1 (Watanabe et al. 1998, Bondurand et al. 2000, Potterf et al. 2000, Takeda et al. 2000, Lang et al. 2005) and signaling pathways such as  $\alpha$ -MSH, cAMP and Wnt (Takeda et al. 2000, Bertolotto et al. 1996, Kamaraju et al. 2002) as well as there being the possibility that it can regulate itself (Steingr msson et al. 2004). However, in zebrafish only Sox10 and Wnt signaling have been shown to be involved in regulating *mitfa* expression (Elworthy et al. 2003, Dorsky, Raible and Moon 2000), although this is not to say that other factors and pathways are not also involved. As will be discussed in more detail in 1.5.2, it has been demonstrated, both in vivo and in vitro, that Sox10 can bind to and activate expression from the mouse and human *MITF* and zebrafish *mitfa* promoters (Bondurand et al. 2000, Lee et al. 2000, Potterf et al. 2000, Verastegui et al. 2000, Elworthy et al. 2003). Similarly, Pax3 binding sites have been identified in mouse and human *MITF* promoters and in vitro and in vivo activation of expression from these promoters by Pax3 has been demonstrated (Bondurand et al. 2000, Lee et al. 2000, Potterf et al. 2000, Verastegui et al. 2000, Watanabe et al. 1998). Further, some mouse and human cell culture studies have found that SOX10 and PAX3 can act synergistically to activate expression from the *MITF* promoter (Bondurand et al. 2000, Potterf et al. 2000). Conversely, others have found that in human cells, activation by SOX10 is not affected by co-expression of PAX3, nor by the loss of the PAX3 binding site (Lee et al. 2000, Verastegui et al. 2000). There is also a potential cAMP response element (CRE) in the *Mitf* promoter (Goding 2000). Further, increased cAMP can alter *Mitf* expression (Bertolotto et al. 1998) perhaps by mimicking signaling through the c-Kit receptor and activation of the MAP kinase pathway (Hou, Panthier and Arnheiter 2000). Wnt signaling also seems to regulate *Mitf* expression; in mouse culture, addition of Wnt-3a will upregulate expression of *Mitf*. Lef-1 is a downstream effector of Wnt signaling and a Lef-1 binding site exists within the human *MITF* promoter to which Lef-1 has been shown to bind (Takeda et al. 2000). By mutating the Lef-1 binding site, Takeda et al. (2000) reduced *MITF* expression demonstrating the significance of Lef-1 in *MITF* regulation. The same group went on to show that, in human melanocyte and melanoma culture, increased LEF-1 and  $\beta$ -catenin will upregulate reporter gene expression from the *MITF* promoter. Further, they showed that the positive effect of Wnt-3a on *MITF* expression can be attenuated by addition of a dominant negative LEF-1, illustrating the importance of LEF-1 as an effector of Wnt signaling in this instance. Additional supporting evidence for this relationship between Wnt signaling and *Mitf* expression comes from zebrafish. Lef1 is expressed in the early zebrafish embryo and in the neural crest at least at 16 hpf (Ishitani et al. 2005, Lee et al. 2006). The *mitfa* promoter also contains Tcf/Lef binding sites which were shown to bind Lef1 in vitro (Dorsky et al. 2000) and again, dominant negative Tcf will repress expression



from a reporter construct containing the *mitfa* promoter. Importantly however, Dorsky et al. (2000) were able to show that this has significance in vivo where mutation of the Tcf/Lef binding sites prevents Lef1 from binding to these sequences and prevents reporter gene expression.

The Mitf protein, like other bHLH-Zip transcription factors, recognises and binds to the sequence CATGTG, called an E-box. In melanocytes, the E-box must be flanked by a 5'T and/or a 3'A for Mitf to bind (Aksan and Goding 1998). The promoters of many melanocyte specific genes such as *Dct*, *Tyrp* and *Tyrosinase* contain an M-box, an 11 bp sequence which contains an E-box. Mitf is able to bind to these M-boxes and this enables it to regulate expression of *Dct*, *Tyrp* and *Tyrosinase* in vitro (Yasumoto et al. 1997, Bentley, Eisen and Goding 1994, Ganss, Schütz and Beermann 1994, Bertolotto et al. 1998). The ability of Mitf to regulate melanocyte differentiation genes can be altered in a number of ways. Phosphorylation of Mitf, via the MAP kinase pathway may alter, positively or negatively, the activity and stability of Mitf (reviewed in Goding 2000). In addition, Mitf binds to M-boxes either alone as a homodimer or as a heterodimer with related partner proteins Tfe3, Tfeb and Tfec (Hemesath et al. 1994) or with various other co-factors. These proteins will also modulate Mitf activity and affect melanocyte differentiation. In mouse, Tfe3 and Tfeb are not expressed at times during development when Mitf is important in melanocytes (Nakayama et al. 1998) and in samples from melanoma cell lines Tfe3 does not coimmunoprecipitate with Mitf (Verastegui et al. 2000) so it seems unlikely that these proteins are important for Mitf activity in melanocytes. Supporting this conclusion, the only described Tfe orthologue in zebrafish, Tfe3a, is not expressed in neural crest at all (Lister et al. 2001). Two other important co-factor proteins, CBP and p300, coimmunoprecipitate with Mitf in samples from mouse melanocyte and melanoma cell lines. These proteins interact with the same part of the Mitf protein as does E1A (Sato et al. 1997). E1A can prevent transcription from the M-box (Yavuzer et al. 1995). Therefore if CBP and p300 prevent Mitf from interacting with E1A, they will also promote transcription from the M-box. Thus, CBP/p300 are Mitf partner proteins that may be important for regulating Mitf activity in melanocytes in vivo. A final example of an Mitf co-factor is Pias3. It is known to repress activation of genes by Mitf (Sonnenblick, Levy and Razin 2004). Zebrafish equivalents for CBP/p300 and Pias3 have not yet been described, demonstrating the considerable gaps in our understanding of gene regulation by Mitf in zebrafish.

### **1.2.1ii Tyrosinase Family Proteins**

We have already mentioned three melanogenic enzymes: Tyrosinase, Dopachrome tautomerase (Dct) and Tyrosinase-related protein 1 (Tyrp1). These three proteins

comprise the tyrosinase family of enzymes and are important in melanin synthesis (Tsukamoto, Jiménez and Hearing 1992). Tyrosinase catalyses two steps in the reaction that converts tyrosine to melanin; the reaction that converts tyrosine to dopachrome and later the reaction that converts dopachrome to dopaquinone (Hearing 1987, Körner and Pawelek 1982). Mutations in this gene lead to albinism, a loss of pigment from the eye and from NC-derived melanocytes in humans (oculocutaneous albinism type 1A, OCA1, OMIM 203100), mice (Shibahara et al. 1990) and fish (Page-McCaw et al. 2004). Melanocytes themselves persist in these mutants; they express other melanocyte specific genes but simply fail to melanise. Dct is one of the earliest markers of melanocyte development both in mouse and in zebrafish (Steel et al. 1992, Kelsh et al. 2000). It is interesting to note that in mouse Tyrosinase and Tyrp1 begin to be expressed substantially later than Dct (Seo et al. 1998) but in zebrafish they are all expressed from 19 hpf (Camp and Lardelli 2001, Kelsh et al. 2000, Thisse and Thisse 2004). Dct catalyses the conversion of dopachrome to DHICA (Jackson et al. 1992). Mice which are mutated at the Dct locus, called slaty, have dark grey coats (Jackson et al. 1992). Tyrp1 also has dopachrome tautomerase activity (Zdarsky et al. 1990) but its exact role is controversial as it also seems to stabilise Tyrosinase and has DHICA oxidase activity (Murisier and Beermann 2006). Mouse mutants in this gene are called brown (Jackson 1988), a reflection of their coat colour. As we have mentioned, there are two kinds of melanin, pheomelanin and eumelanin. Dct and Tyrp1 are most important for eumelanin synthesis; demonstrated by the change in coat colour of mutant mice (Zdarsky et al. 1990) and by the loss of Dct and Tyrp1 expression in agouti mouse melanocytes during their pheomelanogenic phase (Kobayashi et al. 1995).

### **1.2.1iii Other Important Melanocyte Proteins**

Another gene often used as a melanocyte marker is *Silver/Pmel17*, named after the coat colour of mice mutated at this locus (Kwon et al. 1995, Kwon et al. 1991, Martínez-Esparza et al. 1999). Silver is involved in the formation of the intraluminal fibrils in melanosomes on which melanin is polymerized (Berson et al. 2003, Kelly and Balch 2003). In mouse, *Silver* is expressed from very early in the melanocyte lineage both in the PRE and in the neural crest (Baxter and Pavan 2003). Zebrafish have two copies of this gene, *silva* and *silvb*, but only *silva* is expressed in the neural crest (Schonthaler et al. 2005).

All of the above mentioned genes are involved in melanin synthesis or storage. Of course there are other important melanocyte genes. For example the receptor tyrosine kinase c-Kit, and its zebrafish orthologue, kit. *c-Kit* is mutated in White spotting mice, which have white coats or patches of white fur among other phenotypes (Geissler, Ryan and Housman 1988), and in *sparse* zebrafish which have a reduction in melanophores



(Parichy et al. 1999). The c-Kit ligand is Steel factor and signaling through c-Kit and Steel factor is important in melanocyte survival, proliferation and migration (Morrison-Graham and Takahashi 1993, Wehrle-Haller, Meller and Weston 2001, Kelsh et al. 2000, Yoshida et al. 1996, Parichy et al. 1999).

### 1.2.2 Xanthophore Development

Xanthoblasts migrate only on the lateral pathway (Le Douarin and Kalcheim 1999). When fully differentiated they have a delicate, spidery morphology. Xanthophore pigment is made up of pteridines which are yellow, visible from approximately 35 hpf. Pteridines also autofluoresce under fluorescent light. The major published markers for xanthophores are *GTP-cyclohydrolase I (gch)* and *xanthine dehydrogenase (xdh)* (Parichy et al. 1999). They both code for enzymes required for pteridine synthesis. Gch functions in the pathway which converts guanosine triphosphate to tetrahydrobiopterin (BH<sub>4</sub>), a precursor to pteridine pigments. Xdh is required to make xanthopterin, a xanthophore pteridine pigment (Epperlein and Löfberg 1990, Reaume, Knecht and Chovnick 1991). However, a product of Gch activity, BH<sub>4</sub>, is also a co-factor for phenylalanine hydroxylase, which converts phenylalanine to tyrosine (a precursor to melanin), and regulates the activity of Tyrosinase (O'Donnell, McLean and Reynolds 1989, Wood et al. 1995, Nagatsu and Ichinose 1999). This might explain why *gch* is also known to be expressed in early melanoblasts (Parichy et al. 2000b).

### 1.2.3 Iridophore Development

We mention iridophores here only very briefly since they are not a focus of this thesis. Iridoblasts migrate solely on the medial migration pathway (Kelsh 2004). The shiny nature of iridophores is due to the presence of reflective guanine platelets which become visible from approximately 40 hpf. Marker genes for iridophores include *ednrb1*, which is expressed in the embryo but appears only to have a function in the adult fish (Parichy et al. 2000a) and *ltk* which has recently been shown to be key for iridophore specification (Lopes et al. 2008).

## 1.3 Neural Crest Disease and Models

Where neural crest development fails in humans, a number of genetic diseases result. These are collectively known as neurocristopathies. One such disease is Hirschprung's disease, the major symptom of which is aganglionic megacolon resulting from a loss of neural crest derived enteric neurons. Hirschprung's disease is often caused by mutations in the gene *RET* (Badner et al. 1990, Romeo et al. 1994, Edery et al. 1994). Another example of a neurocristopathy is Waardenburg syndrome. This is in fact a group of four syndromes which have different combinations of a number of symptoms

which vary in their severity. All neural crest derivatives can be affected to differing degrees in Waardenburg syndromes. Symptoms always include loss of pigment in the skin, and sometimes deafness (Read and Newton 1997) as a result of loss of melanocytes. WS2 is the simplest of the syndromes since patients only have the pigmentary defects. This syndrome is often linked with mutations in *MITE*, although recently a case has been described where the transcription factor *SOX10* is mutated (Tassabehji, Newton and Read 1994, Hoth et al. 1993, Bondurand et al. 2007). WS1 is characterized by loss of pigment cells with the addition of dystopia canthorum, whilst WS3 patients display these symptoms as well as limb deformities. Thus, WS1 and WS3 affect the formation of neural crest derived craniofacial cartilage as well as melanocytes. These syndromes are associated with mutations in the transcription factor *PAX3* (Tassabehji et al. 1992). The fourth syndrome, WS4, combines the symptoms of Waardenburg syndrome with those of Hirschprung's disease (Omenn and McKusick 1979, Shah et al. 1981). Mutations in several genes have been associated with this syndrome including *endothelin B receptor* (*EDNRB*), its ligand *endothelin 3* (*EDN3*), and *SOX10* (Edery et al. 1996, Attié et al. 1995, Hofstra et al. 1996, Pingault et al. 1998, Kuhlbrodt et al. 1998b).

There are many mutants in both mouse and zebrafish which have defects in neural crest development (<http://www.informatics.jax.org/>, Eppig et al. (2007), Kelsh et al. (1996), Odenthal et al. (1996)). These have enabled researchers to learn a great deal about the biology and genetics of neurocristopathies (Bennett and Lamoreux 2003). The major genes which are mutated in Waardenburg syndromes all have equivalent mouse mutants which provide models for further study of the disease: *microphthalmia/Mitf* (Hodgkinson et al. 1993), *plotch/Pax3* (Chalepakakis et al. 1994), *piebald-lethal/Ednrb* (Hosoda et al. 1994), *lethal-spotting/Edn3* (Baynash et al. 1994) and *Dom/Sox10* (Herbarth et al. 1998, Southard-Smith, Kos and Pavan 1998, Lane and Liu 1984). Similarly, there are zebrafish equivalents for some of these mutants/genes and study of these also contributes to the understanding of Waardenburg syndrome: *nacre/mitfa* (Lister et al. 1999), *rose/ednrb1* (Parichy et al. 2000a) and *cls/sox10* (Dutton et al. 2001).

As mentioned above, humans with WS4 have symptoms encompassing both pigmentary and neural phenotypes; these patients often have lesions at their *SOX10* locus (Pingault et al. 1998). *Sox10* mutant embryos in both mouse and zebrafish mirror many aspects of the human phenotype closely and provide excellent models for study of the human disease (Herbarth et al. 1998, Kelsh et al. 1996, Dutton et al. 2001, Southard-Smith, Kos and Pavan 1998, Lane and Liu 1984). The most studied mouse *Sox10* mutant is Dominant megacolon (*Dom*), a spontaneous mouse mutant identified in 1984 (Lane and Liu 1984, Herbarth et al. 1998). *Sox10<sup>Dom</sup>* homozygous mice die before 13 days

of gestation but mice heterozygous for the mutation show white spotting (white belly spots and white feet) as a result of loss of melanocytes and a loss of enteric neurons (leading to megacolon). Other alleles show similar defects. *Sox10<sup>Hry</sup>* is a mouse line with a transgene insertion that disrupts *Sox10* regulatory sequences (Antonellis et al. 2006). Heterozygous *Sox10<sup>Hry</sup>* mice do not display a phenotype beyond variable white belly spotting. However, homozygous mice survive and have a complete loss of melanophores and a partial loss of enteric neurons. *Sox10<sup>LacZ</sup>* is another transgenic line where *LacZ* is inserted into the *Sox10* coding region. The transgene in this case is homozygous lethal; heterozygous mice display a phenotype similar to the spontaneous *Sox10<sup>Dom</sup>* mutant (Britsch et al. 2001). Zebrafish *sox10* mutants, first called *colourless* (*cls*), are also deficient in all non-skeletogenic neural crest derivatives (Dutton et al. 2001, Kelsh and Eisen 2000). Mouse *Sox10* mutants display haploinsufficiency whereby heterozygous mice display a non-lethal phenotype resulting from loss of function of one gene copy. This heterozygous phenotype can be studied in addition to the embryonic phenotype of homozygous embryos prior to death. One difference between mouse and zebrafish models is that zebrafish *sox10* heterozygotes are essentially indistinguishable from wild types; there is no haploinsufficiency. It is, therefore, only the homozygous embryos which are studied. Like the mouse homozygous *sox10* mutants, homozygous zebrafish mutants also die before reaching adulthood, at approximately 10 dpf. In both organisms there is a loss/reduction in markers for the affected neural crest derivatives (Southard-Smith et al. 1998, Dutton et al. 2001) and increased apoptosis of neural crest derived cells (Kapur 1999, Southard-Smith et al. 1998, Dutton et al. 2001). However, the detailed phenotypes of these different *Sox10* mutants have not been fully explored. For example, we know little about how far non-skeletogenic neural crest derivatives may be able to develop and which genes are expressed in these cells before specification fails. Further exploration of the phenotypes of the *sox10* mutants available to us, particularly at a molecular level, may yield more clues about the precise role that Sox10 plays in the neural crest.

## 1.4 Sox Proteins

Humans and mice have 20 orthologous pairs of proteins which comprise the Sry-like HMG box, or Sox, family of transcription factors (Schepers, Teasdale and Koopman 2002). All have HMG DNA binding domains (Laudet, Stehelin and Clevers 1993) and bind within the minor groove of DNA (Werner et al. 1995). This causes bending of the DNA into an L-shape bringing disparate sections of DNA together (Connor et al. 1994, Ferrari et al. 1992, Werner et al. 1995). This has obvious implications for understanding the regulation of genes where Sox proteins have an involvement since transcription factor binding sites which might normally be widely separated may be

brought closer together. Sox proteins in general may require particular binding partners and often function in co-operation with other transcription factors to induce target gene expression (reviewed in Wegner (2005)). This may impart specificity on a family of proteins which are often expressed to different effect in several cells types but which are very similar in the DNA sequences that they recognise (Kamachi, Uchikawa and Kondoh 2000, Wilson and Koopman 2002).

The Sox family is categorized into ten groups, A-J, according to similarities in their HMG domain sequences (Schepers et al. 2002). The SoxE group comprises Sox8, Sox9 and Sox10. These proteins can function as monomers but may also be required to be dimerised to function properly in some situations (Wegner 2005, Peirano and Wegner 2000, Schlierf et al. 2002). Another interesting feature of the genes in this family is that they have both nuclear and cytoplasmic localization sequences (Gasca et al. 2002, Poulat et al. 1995, Südbek and Scherer 1997). In the case of Sox10, it has been shown in mouse cell culture that the protein is shuttled between the nucleus and the cytoplasm and can be sequestered in the cytoplasm (Rehberg et al. 2002). Thus a cell may still have detectable SoxE protein but it is not functional since it is not present in the correct part of the cell. However, there is as yet no evidence that this occurs in zebrafish.

### 1.4.1 Sox8

In mouse, Sox8 is expressed in the brain, more specifically in glia. It is mutated in patients with ATR-16, a disease characterised by mental retardation (Cheng et al. 2001, Pfeifer et al. 2000). Other sites of expression in mouse include premigratory neural crest and neural crest derivatives such as the branchial arches, DRGs and the peripheral nervous system as well as the otic placode, cranial ganglia, limb buds and spinal cord (Sock et al. 2001). In chick, Sox8 is expressed in many of the same cell types, such as premigratory neural crest, DRGs, enteric neurons and developing limbs (Bell, Western and Sinclair 2000). The described zebrafish homologue (Cresko et al. 2003) is not expressed in neural crest derivatives (Yan et al. 2005).

The exact function of Sox8 has not been described convincingly in any organism. A targeted mouse mutant displays a general reduction in weight, a phenotype which is not particularly informative when elucidating the function of Sox8. The only specific effect upon any of the cell types in which Sox8 is known to be expressed is in Sertoli cells where mutations in *Sox8* cause a deregulation of spermatogenesis and progressive male infertility (Sock et al. 2001, O'Bryan et al. 2008). It is suggested that the lack of specific phenotypes in *Sox8* mutants is as a result of compensation for the loss of functional Sox8 by other genes in the SoxE family. Mice that are

heterozygous for mutations in both *Sox8* and *Sox10* have a phenotype more like that of a *Sox10* homozygous mutant which supports the idea that there may be potential for functional redundancy between SoxE genes (Maka, Stolt and Wegner 2005). In chick, overexpression of *Sox8* can induce neural crest (Cheung and Briscoe 2003). However, as we mentioned previously, Sox proteins have rather generic binding sites and all of the genes involved in neural crest induction regulate one another, and since *Sox8* is not expressed in presumptive neural crest it is unlikely to play a role in the formation of normal neural crest.

### 1.4.2 Sox9

*Sox9* expression in mouse is mostly associated with chondrogenesis but expression is also present in structures such as the heart, notochord and the testes (Wright et al. 1995, Wagner et al. 1994). In humans, mutations in *Sox9* result in the syndrome Campomelic Dysplasia symptoms of which include bone malformations and sex reversal (Foster et al. 1994). Heterozygous *Sox9* mouse mutants display similar phenotypes to those described for Campomelic Dysplasia (Bi et al. 2001, Mori-Akiyama et al. 2003). Homozygous mutations in *Sox9* are lethal in mouse. A number of targets for *Sox9* have been identified which, consistent with a role in chondrogenesis, include *AMH* (Anti-Mullerian Hormone), *Col2a1* and *Col11a2* (see Marshall and Harley (2000) for review). Zebrafish have two homologues for *Sox9*, *Sox9a* and *Sox9b*, the expression patterns of which both overlap with and complement one another to make up the same expression pattern as their mouse counterpart (Chiang et al. 2001). *Sox9a* is expressed in the brain, craniofacial cartilage, testes, ear, eye, somites and fins (Yan et al. 2005). *Sox9b* is expressed in the brain, sensory organs and fins as well as during chondrogenesis (though at a later stage than *Sox9a*). The zebrafish mutant for *Sox9a*, *jellyfish*, displays cartilage and fin defects (Yan et al. 2002). *Sox9b* zebrafish mutants also have cartilage and fin defects, though less severe (Yan et al. 2005). The effect of generating embryos mutant for both genes is additive and strongly resembles the mouse *Sox9* mutant phenotype again providing supporting evidence for a role for *Sox9* in chondrogenesis (Yan et al. 2005).

*Sox9* is also expressed in mouse presumptive neural crest; evidence of a role for *Sox9* in this transient population of cells (Li et al. 2002). This was demonstrated in chick through the induction of crest markers by overexpression of *Sox9* (Cheung and Briscoe 2003). Loss of function analysis in zebrafish (either as a result of mutation or morpholino knockdown) shows a strong reduction in the expression domains of early crest markers *snailb*, *foxd3* and *sox10* (Yan et al. 2005). Gain of function experiments, by mRNA injection, show that both *sox9a* and *sox9b* can induce ectopic expression of *foxd3*, *sox10* and *snailb* (Yan et al. 2005). These results support an additional role for

*Sox9* genes in zebrafish neural crest formation.

## 1.5 Sox10

### 1.5.1 Sox10 Expression

*Sox10* in *Xenopus* is expressed in the neural crest even before closure of the neural tube (Aoki et al. 2003). In human, mouse, zebrafish and chick it is not expressed until later, in the premigratory neural crest (Southard-Smith, Kos and Pavan 1998, Dutton et al. 2001, Cheng et al. 2000, Pusch et al. 1998, Bondurand et al. 1998). Other sites of *Sox10* expression include the ear and oligodendrocytes (Aoki et al. 2003, Southard-Smith et al. 1998, Cheng et al. 2000). We will concentrate on the expression in the neural crest (Dutton et al. 2001). Expression of *Sox10* persists in migrating neural crest cells including enteric neuron precursors and melanoblasts (Kuhlbrodt et al. 1998a, Dutton et al. 2001, Southard-Smith et al. 1998, Cheng et al. 2000, Aoki et al. 2003). However, in most cell types, probably including zebrafish melanophores, *Sox10* expression is gradually lost as cells differentiate (Britsch et al. 2001, Dutton et al. 2001, Sonnenberg-Riethmacher et al. 2001, Cheng et al. 2000, Aoki et al. 2003, Kim et al. 2003). In glia, *Sox10* expression persists into adulthood (Kuhlbrodt et al. 1998a, Britsch et al. 2001, Sonnenberg-Riethmacher et al. 2001, Southard-Smith et al. 1998, Cheng et al. 2000, Bondurand et al. 1998, Pusch et al. 1998). Persistent *Sox10* expression may also be a feature of melanocytes in mouse. *Sox10* expression has been reported in vivo in melanocytes during late embryonic stages and after birth, although it is downregulated in melanocyte stem cells (Osawa et al. 2005). There are conflicting reports about whether *Sox10* is consistently expressed in melanocyte and melanoma cell lines (Southard-Smith et al. 1998, Cook et al. 2005).

### 1.5.2 Proposed Functions for Sox10

As has been outlined, Sox10 is expressed in many different cell types some of which also express other SoxE family genes. What is it then that Sox10 is important for in these different cell types? What is its function? In fact, it would appear that Sox10 has many different roles within the embryo depending upon the context of its expression. Some of the current thinking about these roles is outlined below.

In *Xenopus* and chick there is evidence that Sox10 has a very early role in the specification of cells to become neural crest. In both organisms, overexpression of Sox10 in embryos results in increased expression of neural crest markers e.g. *slug* (Aoki et al. 2003, Cheung and Briscoe 2003). Additionally, in *Xenopus* there is an increase in pigmented melanocytes, although overexpression of *slug* by mRNA injection has the same effect. In support of this relationship, morpholino knockdown



of *Sox10* in *Xenopus* results in a reduction in the expression of *slug* and so a reduction in neural crest (Honoré, Aybar and Mayor 2003). However, there is no failure of neural crest formation in either mouse or zebrafish *Sox10* mutants (Dutton et al. 2001, Britsch et al. 2001) so it would appear that an early role for Sox10 in the neural crest, proposed principally from work in frog, does not extend to other organisms. On the other hand, it has been proposed that there are variations in the potential for functional redundancy between the SoxE genes between species so that altering levels of any of those genes will have different effects in different organisms (Kelsh 2006). In addition, we discussed above that expression of many of the genes involved in early crest induction/specification are dependent on each other. It is easy to imagine, therefore, that overexpression of Sox10 does not directly promote formation of neural crest but that it could alter the expression patterns of other genes, for example *Sox9*, which in turn affect the formation of the neural crest. In chick and zebrafish, *Sox9* is expressed ahead of *Sox10*, consistent with Sox9 having a primary role in neural crest formation and a role for Sox10 being slightly later in development (McKeown et al. 2005). These results indicate that Sox10 may be involved in the neural crest gene regulatory network but they do not define its role.

Other roles for Sox10 later in neural crest development have also been put forward. Increased cell death observed in *Sox10* mutants (Southard-Smith et al. 1998, Dutton et al. 2001, Kapur 1999, Paratore et al. 2001, Sonnenberg-Riethmacher et al. 2001) has led some to suggest a role for Sox10 in undifferentiated neural crest cell survival (Mollaaghababa and Pavan 2003, Wegner 2005, Paratore et al. 2001). Indeed, morpholino knockdown of Sox10 in *Xenopus* leads to increased apoptosis and decreased proliferation in the neural folds, again supporting the hypothesis that Sox10 has a role in neural crest maintenance. Alternatively, it could be that these results occur as an indirect effect of the role of Sox10 in specification of neural crest cells to their respective fates. Thus, in the absence of Sox10, neural crest cells fail to become specified and, as has been demonstrated in zebrafish, die by apoptosis (Dutton et al. 2001, Kapur 1999, Southard-Smith et al. 1998). A role for Sox10 in fate specification has been demonstrated in multiple cell types and is particularly well supported in the case of melanocytes. *Mitf* is the master regulator of melanocyte differentiation (reviewed Levy, Khaled and Fisher (2006), Goding (2000)) and Sox10 mutants fail to express it (Britsch et al. 2001, Dutton et al. 2001, Potterf et al. 2001). A test of whether Sox10 could regulate *Mitf* came from experiments in cell culture that showed that expression of either mouse or zebrafish Sox10 in mammalian cell-lines could activate transcription from the *Mitf* promoter (Lee et al. 2000, Verastegui et al. 2000, Bondurand et al. 2000, Potterf et al. 2000, Elworthy et al. 2003). These same authors used gel shift assays to demonstrate that Sox10 can directly bind the *Mitf*

promoter. The ease of analysis of reporter expression in transient transgenic zebrafish allowed the demonstration *in vivo* that at least one functional Sox10-binding site in the *mitfa* promoter is necessary for *mitfa* expression in the neural crest (Elworthy et al. 2003). In summary, in melanocytes Sox10 drives expression of *mitfa*, the key gene for a cell to become a melanocyte, and so Sox10 has an important role in melanocyte fate specification.

A similar specification role has been described in autonomic neurons, where Sox10 induces expression of *Phox2b*, and in DRG sensory neurons where it induces expression of *ngn1*. *Phox2b* is a homeobox transcription factor which, together with *Mash1*, is important in autonomic neuron specification (Guillemot et al. 1993, Lo, Tiveron and Anderson 1998, Pattyn et al. 1999). It has a direct role in regulating neuron differentiation genes such as *tyrosine hydroxylase* (Pattyn et al. 1999, Lo et al. 1998). Kim et al. (2003) showed that in mouse sympathetic neurons, Sox10 is required to activate expression of *Phox2b* and *Mash1*. They found that most Sox10 positive cells near the dorsal aorta in a wild type mouse are also *Phox2b* and *Mash1* positive and the equivalent cells in *Sox10* mutant embryos are not *Phox2b*/*Mash1* positive. This suggested that Sox10 is required for induction of *Phox2b*/*Mash1* and thus to promote neuron specification. However, Sox10 alone is not able to increase expression of *Mash1* suggesting that the relationship between Sox10 and *Mash1* may not be direct. Instead, the authors found that BMP2 induces expression of *Mash1* and the inhibition of this action by TGF $\beta$  can be prevented by constitutive expression of Sox10 (Kim et al. 2003). In fish, morpholino knockdown of *phox2b* results in a loss of enteric neurons. The expression of *Phox2b* in these neurons, and so specification of enteric neurons, is also dependent upon Sox10. Again however, expression of *phox2b* is not necessarily directly activated by Sox10 (Elworthy et al. 2005).

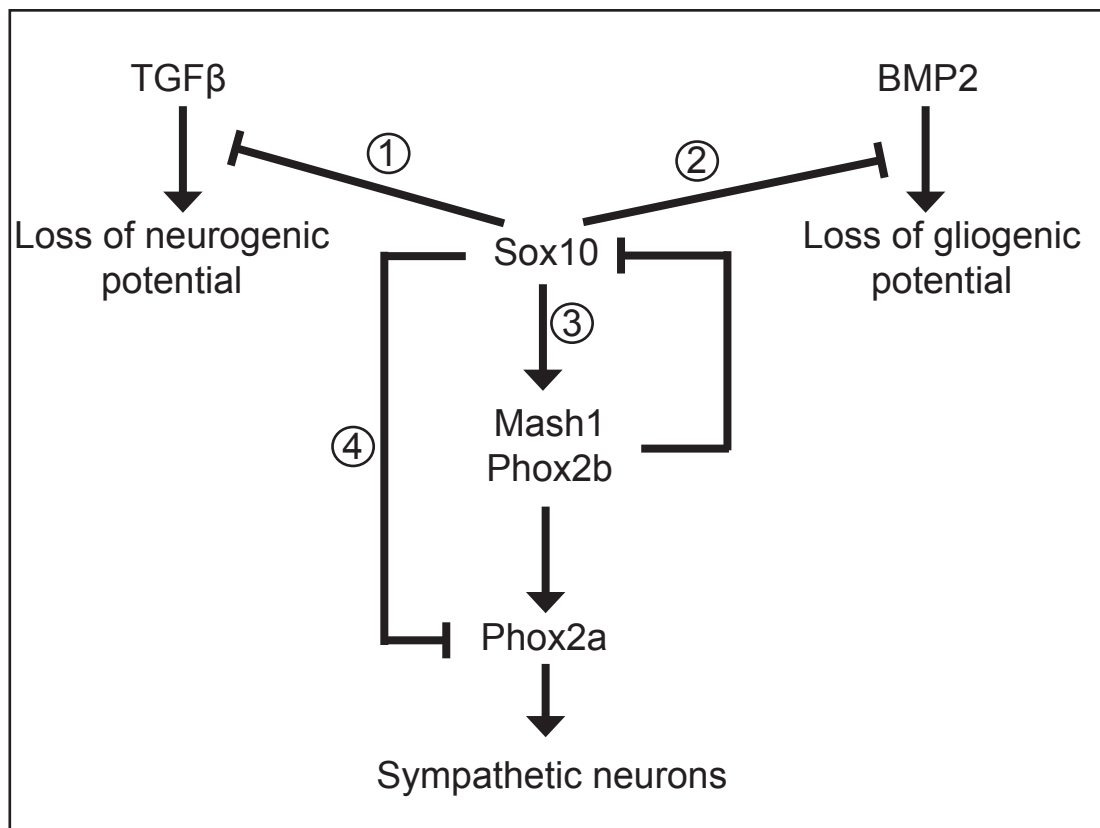
In mouse *Sox10* mutants, DRG sensory neurons form but later degenerate (Britsch et al. 2001). In zebrafish *sox10* mutants DRGs are severely disrupted, being reduced in number, misplaced in the trunk and absent from the tail (Britsch et al. 2001, Kelsh and Eisen 2000). A study of glial defects in mouse DRGs was interpreted to suggest that Sox10 did not act directly in the sensory neuron lineage. They reasoned that since glia completely fail to differentiate in *Sox10* mutants (Britsch et al. 2001, Paratore et al. 2001, Carney et al. 2006) sensory neurons die in the DRGs because of lack of glial trophic support (Britsch et al. 2001). However, a recent paper re-assessed this issue in the zebrafish model and demonstrated convincingly that Sox10 is actually required transiently in sensory neurons (Carney et al. 2006). Furthermore, the authors show that its role is in fate specification of the sensory neuron lineage. *ngn1* is a gene encoding a pivotal transcription factor in sensory neurons and it has been shown that



Sox10 is able to regulate its expression (Carney et al. 2006). Tests of whether the regulation of *ngn1* by Sox10 is direct have yet to be performed, but overexpression studies in the zebrafish embryo indicate that *ngn1* transcription is activated rapidly after, and thus perhaps as a direct response to, Sox10 expression. Whether other sensory neuron genes are directly regulated by Sox10 remains to be assessed, although *sox10* expression disappears rapidly from this lineage and so a late role in differentiation or fate maintenance is unlikely.

In contrast, a late role for Sox10 is much more likely in glia as expression persists in this cell type into adulthood (Bondurand et al. 1998, Kuhlbrodt et al. 1998a, Pusch et al. 1998, Southard-Smith et al. 1998, Britsch et al. 2001, Sonnenberg-Riethmacher et al. 2001, Cheng et al. 2000). A requirement for Sox10 has been demonstrated in this cell type since no glial markers are ever detected in *Sox10* mutants (Carney et al. 2006, Britsch et al. 2001), nor can glia be encouraged to differentiate in culture from Sox10 deficient neural crest cells (Paratore et al. 2001). It is not clear whether this demonstrates a requirement for Sox10 in glial fate specification or suggests a continuing role in glial differentiation. Of course, these options are not mutually exclusive. Krox20 (Egr2) is a zinc finger transcription factor important in myelination of Schwann cells (Topilko et al. 1994). It has been shown to induce expression of myelin genes such as *Myelin Protein Zero (MPZ)*, *Connexin32* and *MAG* (Nagarajan et al. 2001) and when mutated in mice it prevents promyelination (Topilko et al. 1994). Sox10 is able to induce expression of a reporter gene attached to a *Krox20* regulatory element (Ghislain and Charnay 2006) indicating that it is likely to be a regulator of *Krox20* and thus glial fate specification. Overexpression of Sox10 in cells expressing MPZ ( $P_0$ ) will greatly increase the levels of MPZ expression (Peirano et al. 2000). This upregulation of MPZ may occur via Krox20 but the relationship may be more direct since Sox10 binding sites are present in the promoter region of *MPZ*. In either case, this evidence would support the suggestion of an additional role for Sox10 in glial differentiation. Similarly, the *Connexin32* promoter also contains Sox10 binding sites and in cell culture, addition of Sox10 will increase the expression of *Connexin32* (Bondurand et al. 2001). These examples provide support for a dual role for Sox10 in glia, in both glial differentiation and in glial specification.

One study we mentioned demonstrated that Sox10 is important in sympathetic neuron development via *Phox2b* and *Mash1* (Kim et al. 2003). The same authors also demonstrated that Sox10 performs a contradictory role, one of maintaining crest cells in a stem cell-like state, in this cell type (Figure 1.03). They showed that BMP2 promotes loss of glial potential in neural crest and TGF $\beta$  promotes loss of neurogenic potential. Overexpression of Sox10 can prevent BMP2 and TGF $\beta$  from having these effects,



**Figure 1.03 Roles of Sox10 in sympathetic neuron differentiation**

Sox10 has many roles in sympathetic neuron development (Kim et al. 2003). It is able to maintain neural crest cells in a stem cell-like state by inhibiting the actions of TGFβ and BMP2 (1 and 2). These factors promote the loss of neurogenic and gliogenic potential respectively so by blocking their action, Sox10 maintains neurogenic and gliogenic potential in neural crest cells. Sox10 also acts to promote differentiation by inducing expression of sympathetic neuron specification genes, *Mash1* and *Phox2b* (3). This interaction is not direct but Sox10 is certainly permissive for BMP2 induced expression of *Mash1*. Simultaneously, Sox10 inhibits the differentiation of these cells by repressing expression of *Phox2a* (4).

thus maintaining neurogenic and gliogenic potential and keeping neural crest cells in a stem cell-like state. By similar overexpression of Sox10 in neural crest cells Kim et al. (2003) showed that Sox10 must be downregulated to allow the full differentiation of neural crest cells into neurons. This is because, when present at high levels, Sox10 is able to repress the expression of *Phox2a*, another neuron specific gene and inhibit differentiation. Thus, Sox10 functions in several guises in sympathetic neurons. It is able to simultaneously promote sympathetic neuron specification, prevent loss of gliogenic and neurogenic potential and inhibit differentiation, in so doing, keeping neural crest cells in a stem cell like state.

### 1.5.3 Role of Sox10 in Melanophores

Many different functions for Sox10 have been described in different neural crest derivatives and it can even have many different functions just within one cell type, sympathetic neurons. However, we still do not fully understand the role that Sox10 has within genetic regulatory networks involved in neural crest development. The work presented in this thesis will focus on the role of Sox10 in melanoblasts and melanocytes. We outline below the current understanding of the role of Sox10 in these cells and highlight the areas where we hope to add to this understanding.

One function that Sox10 performs in melanoblasts, one that is not disputed, is to switch on expression of *Mitf* (see 1.5.2). Both mouse and zebrafish *Sox10* mutants fail to express *Mitf* or *mitfa* respectively (Britsch et al. 2001, Dutton et al. 2001, Potterf et al. 2001) and further, researchers have shown that Sox10 can bind to, and activate expression from, the mouse and human *MITF* and zebrafish *mitfa* promoters in vivo and in vitro (Bondurand et al. 2000, Lee et al. 2000, Potterf et al. 2000, Verastegui et al. 2000, Elworthy et al. 2003). We outlined in 1.2.1i some other factors that will also modulate *Mitf* expression (reviewed Goding (2000)). Transcription factors such as Pax3 and Lef-1 are known to bind to and regulate expression from the mouse *Mitf-M* promoter in vitro and in vivo (Watanabe et al. 1998, Bondurand et al. 2000, Potterf et al. 2000, Takeda et al. 2000, Lang et al. 2005) and pathways such as  $\alpha$ -MSH, cAMP and Wnt signaling are required for expression of *Mitf-M* (Takeda et al. 2000, Kamaraju et al. 2002). In addition, co-factors such as CBP/p300 will modulate the activity of the Mitf protein (Sato et al. 1997). This reminds us that relationships between genes are not necessarily single linear relationships but many factors may influence the expression and activity of any given protein. Development involves networks of genetic interactions of which only a small part is usually studied at any one time. It is therefore important to keep in mind that the relationship between Sox10 and Mitf will be modified by other factors which are often not being directly studied.

Of course, once *Mitf* is expressed, this master regulator is able to induce expression of many melanocyte specific genes and cause differentiation. Further to the data described in 1.2.1i illustrating that *Mitf* alone is sufficient to cause melanocyte differentiation in vitro and in vivo (Tachibana et al. 1996, Planque et al. 1999, Béjar, Hong and Scharlt 2003, Lister et al. 1999), there is evidence that *Mitf* alone is able to directly induce expression of many melanocyte specific genes. The promoters of all of the *tyrosinase* family genes have M-boxes and expression from these promoters can be activated by *Mitf* (Budd and Jackson 1995, Bentley, Eisen and Goding 1994, Ganss, Schütz and Beermann 1994, Yasumoto et al. 1994, Yokoyama et al. 1994, Jackson et al. 1991, Yasumoto et al. 1997, Lowings, Yavuzer and Goding 1992, Bertolotto et al. 1998, Camp et al. 2003). Also, in zebrafish, overexpression of *mitfa* can induce expression of *dct* (Lister et al. 1999). *Silver* expression is absent from mouse *Mitf* mutants (Baxter and Pavan 2003) suggesting that *Mitf* can also regulate *Silver*. Direct activation of the *Silver* promoter by *Mitf* was confirmed by both in vitro and in vivo work (Du et al. 2003).

If *Sox10* is able to induce expression of *Mitf*, which in turn is sufficient for melanocyte differentiation, then it could be that this is the only role for *Sox10* in the melanocyte. Work by Elworthy et al. (2003) showed that in zebrafish it is possible to rescue melanophores in *sox10* mutant fish by introduction of *mitfa* RNA into the one cell stage embryo. These rescued cells are fully pigmented, morphologically normal and are able to migrate normally. Elworthy et al. (2003) also demonstrated that driving expression of *mitfa* in the neural crest was quantitatively as effective at rescuing melanophores in either *sox10* or *mitfa* mutants. This result suggests, at least in fish, that *Sox10* is not necessary for melanophore development beyond the requirement for it to activate expression of *mitfa*. Although expression of melanogenic enzymes was not directly assessed, the fact that the rescued melanophores pigment successfully suggests that *Sox10* is not required for expression of melanogenic enzymes in zebrafish. Conversely, in mouse it seems that *Sox10* is required in addition to *Mitf* to achieve *Tyrosinase* expression and subsequent pigmentation (Hou, Arnheiter and Pavan 2006). Hou et al. (2006) found that when cultured cells which did not have functional *Sox10*, and therefore did not express *Mitf*, were made to express *Mitf* independent of *Sox10* these cells still failed to pigment. On closer inspection it was apparent that these cells were expressing melanocyte differentiation proteins such as *Dct* and *Tyrp1* but stopped short of expressing *Tyrosinase* and becoming pigmented. However, if these cells are made to express wild type *Sox10* by viral infection they express *Mitf* and all of the melanocyte proteins examined, including *Tyrosinase*, and they pigment. Thus, the authors suggest that a feed-forward loop must exist where *Sox10* activates *Mitf*, which alone will activate most melanocyte differentiation genes but which must act in conjunction with *Sox10* to activate *Tyrosinase* expression. Indeed, the *Tyrosinase*

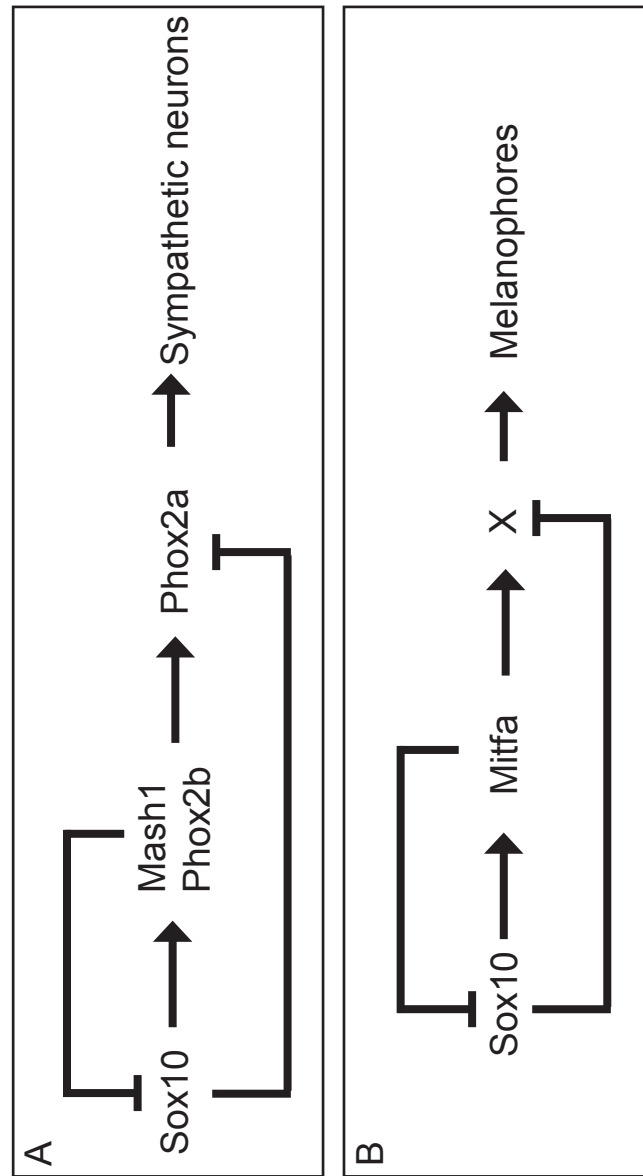
promoter does possess a Sox10 binding site (Murisier, Guichard and Beermann 2007). However, the work of Hou et al. (2006) was conducted in vitro. Primary neural crest was cultured for 10 days after viral infection before assaying. This is a considerable period of time in culture and does not reflect the timing of melanocyte gene expression and development in vivo. It would be valuable therefore, to support this work with in vivo data before drawing firm conclusions as to whether Sox10 is necessary in mouse melanocytes beyond the requirement for it to activate *Mitf* expression.

Supporting the hypothesis of a later role for Sox10 in melanocytes beyond switching on *Mitf* expression, Sox10 has also been implicated in the activation of *Dct* expression. Sox10 can activate reporter gene expression from mouse and human *Dct* promoter sequences (Britsch et al. 2001, Potterf et al. 2001). Two groups have gone further to show that in vitro, Sox10 is able to activate expression of *Dct* in both human and murine cell lines and they have identified the sites at which Sox10 binds the *Dct* promoter (Jiao et al. 2004, Ludwig, Rehberg and Wegner 2004). Activation of *Dct* expression by Sox10 can be enhanced further by also expressing *Mitf* in the same cells (Jiao et al. 2004, Ludwig et al. 2004). Again, this work was carried out in vitro and remains to be supported by in vivo data. However, Hou et al. (2006) found that whilst Sox10 may be able to induce *Dct* expression it is not required for *Dct* expression; *Dct* can be induced by expression of *Mitf* in Sox10-deficient primary neural crest culture. In zebrafish, a late role for *Sox10* in melanophore differentiation certainly seems unlikely from the evidence of Elworthy et al. (2003) and since *Sox10* has been briefly noted as being downregulated in melanophores as they differentiate (Dutton et al. 2001). Data from human and mouse cell culture about whether Sox10 expression persists in melanocytes is inconclusive. In mouse B16 melanoma cell lines and in human melanomas, Sox10 is expressed in significant amounts in differentiated cells (Kamaraju et al. 2002, Khong and Rosenberg 2002). Of course these studies come with the caveat that cell culture, particularly when using altered cell lines such as melanomas, is not necessarily representative of in vivo events. In an in vivo study, Sox10 was seen to be downregulated in melanocyte stem cells in the hair follicle but was also maintained in differentiating cells in the epidermis at least until E18.5 and in the hair follicle until P6 (Osawa et al. 2005). However, there are some significant gaps in the timecourse of the data from this paper, for example they do not look at expression after P6. Conversely to the data above, in human primary cell lines Sox10 has been shown to be downregulated in differentiating melanocytes (Cook et al. 2005).

Hou et al. (2006) postulate that the differences we see in the data we have to date from mouse and zebrafish about the role of Sox10 in melanocytes are due to actual interspecies differences. However, the emerging picture from mouse is neither

consistent nor complete since a significant amount of data has been gathered in vitro, often in abnormal melanoma cell lines, and remains to be tested in vivo. There is also more to be learnt from zebrafish about exactly which genes Sox10 and Mitfa are able to regulate in this organism. We suggest that there is currently insufficient data, from either model organism, to confirm that differences observed between the apparent function of Sox10 in melanocytes versus melanophores are due to interspecies differences. We would like to use zebrafish to try to reconcile some of the data surrounding Sox10 function in melanocyte differentiation including investigating which genes Sox10 and Mitfa are able to regulate.

Given the many roles for Sox10 in the neural crest that we discussed in 1.5.2, it seems unlikely that its role in melanophores will be as simple as just switching on *mitfa*. A model proposed by Kim et al. (2003) for Sox10 function in sympathetic neuron differentiation is presented in Figure 1.04 and we would like to test whether Sox10 might function in a similar genetic network in melanophores. Kim et al. (2003) provide both in vitro and in vivo data to convincingly support their model. However, the authors do not claim to have demonstrated direct relationships between the constituent genes and this remains the only paper relating to this model. Kim et al. (2003) demonstrated that Sox10 is required for induction of *Mash1* and *Phox2b* thus promoting neuron differentiation (see 1.5.2). However, they also found that this requirement may only be transient. Early in development Sox10 positive cells in a position appropriate for sympathetic neurons are often also *Mash1* and *Phox2b* positive but at later stages *Mash1* and *Phox2b* positive cells are not Sox10 positive. This pattern of expression also suggested that persistent expression of Sox10 may actually disrupt neuronal differentiation. In fact, the group went on to provide evidence that overexpression of Sox10 can inhibit neuronal differentiation through repression of genes downstream of *Mash1* and *Phox2b*. Firstly, in wild type mice the expression patterns of Sox10 and *Phox2a*, a sympathetic neuron protein downstream of *Mash1* and *Phox2b*, are mutually exclusive. Secondly, in contrast to *Mash1* mutant mice which lack *Phox2a* expression entirely, *Sox10* mutant mice exhibit a derepression of *Phox2a*, the neuronal marker HuD and potentially many other neuronal markers. Further, by overexpression of Sox10 in neural crest cells Kim et al. (2003) showed that these cells cannot fully differentiate into neurons until Sox10 is downregulated. They also showed that overexpression of *Mash1* or *Phox2b* in vitro reduces expression of Sox10. This suggests that in vivo a negative feedback loop may exist whereby Sox10 is downregulated by *Mash1* and *Phox2b* to allow neuron differentiation. Another layer of complexity is added to this model because the opposing functions of Sox10 to promote and inhibit neuronal differentiation are differentially affected by levels of Sox10. Thus, whilst expression of *Mash1* and *Phox2b* only require low levels of Sox10 (they are still expressed in



**Figure 1.04 Models for Sox10 function in mouse autonomic neurons and zebrafish melanophores**

A. Figure adapted from Kim et al. 2003 showing their model for Sox10 function in autonomic neurons.

B. Model, based on A, for the role of Sox10 in zebrafish melanophores.



heterozygous mutants), inhibition of *Phox2a* requires much higher levels of Sox10 (derepression is observed in heterozygous and well as homozygous mouse mutants).

We can draw clear parallels between the detailed model of Sox10 function in sympathetic neurons and what is already known about Sox10 function in melanophore differentiation. Equivalent to the requirement of Sox10 for *Mash1* and *Phox2b* expression in sympathetic neuron development, it has been shown that Sox10 is required in melanophores for expression of *mitfa* (Elworthy et al. 2003). Of course the difference here is that this has been demonstrated to be a direct relationship (Elworthy et al. 2003). In zebrafish melanophores the requirement for Sox10 may also be transient. By expressing *mitfa* independent of Sox10 Elworthy et al. (2003) were able to rescue melanophores to the same extent in both *sox10<sup>ts3</sup>* and *mitfa<sup>w2</sup>* mutants suggesting that the requirement for Sox10 in melanophores is only to turn on *mitfa* and thus need only be transient. Data presented in Dutton et al. (2001) suggests that *sox10* is in fact downregulated in differentiating melanophores, as it is in sympathetic neurons, providing anecdotal evidence to support the hypothesis that Sox10 may only be required transiently. The question that arises therefore, is how far do these parallels continue? Is it also the case that, as in sympathetic neuron development, Sox10 must be downregulated in melanophores to allow differentiation to proceed? What is the effect of loss of functional Sox10 on the expression of genes downstream of Mitfa? Are they derepressed as *Phox2a* is in sympathetic neurons? What is the effect on other pigment cell types?

Thus, we propose a testable model, based on that of Kim et al. (2003), for the role of Sox10 in zebrafish melanophores (Figure 1.04). In this model, based on the fact that we know Sox10 is required to activate expression of *mitfa* (Elworthy et al. 2003), Sox10 will promote differentiation but it also acts to repress expression of genes downstream of Mitfa and inhibit differentiation. Therefore, Mitfa is later required to downregulate expression of Sox10 so that repression of downstream genes is lifted and the cells are allowed to develop as melanophores. Importantly this model makes a number of testable predictions. The first prediction from the model is that Sox10 will repress melanophore differentiation so that in *sox10* mutants there will be derepression of genes downstream of Mitfa, a prediction we will test in Chapter 3. The second and third predictions are that Sox10 will be downregulated during melanophore development and that this downregulation is necessary for melanophores to differentiate (see Chapter 4). The fourth prediction is that downregulation of Sox10 will be controlled by Mitfa (see Chapter 5). This work will be important not only in understanding Sox10 function in melanophores but also because it tests the generality of Kim et al.'s model of Sox10 function in neural crest development.



## 1.6 Aims

To test a model for Sox10 function in melanophores by:

- Examining closely the effect of loss of Sox10 and of Mitfa on melanophore and xanthophore marker gene expression
- Examining in detail the expression of Sox10 mRNA and protein in differentiating melanophores
- Testing whether Sox10 must be downregulated to allow melanophores to differentiate
- Assessing the likely direct and indirect requirements for Sox10 and Mitfa in regulating zebrafish melanophore differentiation genes

# Chapter 2

## Materials & Methods

### 2.1 Materials

All reagents were sourced from Sigma unless otherwise stated. Restriction enzymes were purchased from Promega or New England Biolabs (see Appendix). Plasmid vectors used for generation of mRNA or for ISH probe synthesis (together with details of the appropriate enzymes) are in Table 2.01, plasmids used for other purposes are described in the relevant chapters.

### 2.2 Fish Husbandry

Wild type (AB), mutant (*mitfa*<sup>w2</sup>, *sox10*<sup>β3</sup>, *sox10*<sup>β3</sup>;*mitfa*<sup>w2</sup>) and transgenic (*mitfa*(0.9)::GFP) and Tg(-7.2*sox10*:EGFP) zebrafish, *Danio rerio*, were kept in the aquarium at the University of Bath. Natural crosses were set up between fish overnight and the embryos collected in the morning. Embryos were placed in embryo medium (see Appendix) and grown at 28.5 °C. They were staged according to Kimmel et al. (1995). Where embryos were to be manipulated between laying and hatching Watchmakers' No5 forceps were used to dechorionate the embryos. Embryos older than 15 hpf which were to be manipulated in any way were anaesthetised with Tricaine (Ethyl 3-aminobenzoate methanesulphonate, 4 g/L stock, final concentration approximately 0.2 % ((v/v))). Where appropriate, melanisation was inhibited using PTU (1-phenyl-2-thiourea) from 24 hpf at a final concentration of 0.0015 % ((v/v)) in embryo medium.

### 2.3 Histology

#### 2.3.1 Antibody Staining

Embryos were fixed in 4 % (v/v) Paraformaldehyde in PBS (Phosphate buffered saline, Oxoid) overnight at 4 °C. They were washed three times for 5 minutes in PB-Triton (0.1 % ((v/v)) Triton X-100 in PBS) and three times for one hour in MilliQ

Table 2.01 Plasmids and enzymes used for RNA and ISH probe synthesis

Name/gene	Origin	To linearise	Polymerase	Notes
HS- <i>sox10</i>	RNK	Asp718	SP6	
HS- <i>sox10</i> <sup>(L142Q)</sup>	RNK	Asp718	SP6	Sequence of <i>sox10</i> <sup>m618</sup> mutant
CS2+ <i>mitfa</i> <sup>WT</sup>	Dr J Lister	NotI	SP6	
CS2+ <i>mitfa</i> <sup>w2</sup>	Dr J Lister	NotI	SP6	Sequence of <i>mitfa</i> <sup>w2</sup> mutant
pCS-TP	Dr K Kawakami	NotI	SP6	
TYR	Michael Lardelli	SacII	SP6	Camp and Lardelli (2001)
<i>tyrplb</i>	ERG	EcoRI	T3	Obtained as clone number 6894514 from Geneservice, GenBank reference CB353867 and subcloned as an EcoRI/XhoI fragment into Bluescript
<i>silva</i>	Zebrafish International Resource Centre, cb397	NotI	SP6	Thisse et al (2001)
<i>dct</i>	RNK	EcoRI	T7	
<i>sox10</i>	RNK	SalI	T7	
<i>mitfa</i>	Dr J Lister	EcoRI	T7	
<i>gch</i>	Dr D Parichy	SalI	SP6	
<i>xdh</i>	Dr D Parichy	XhoI	T3	
<i>paics</i>	RNK	-	-	Plasmid and probe generated by Chipperfield and Nelson (University of Bath)
<i>CFP</i>	ERG	SalI	T7	Subcloned as an XhoI/NheI fragment into Bluescript KS between XhoI and XbaI.

water. Embryos were incubated in block (1 % (v/v) DMSO, 5 % ((v/v)) horse serum in PB-Triton) for between 2 and 3 hours. They were then incubated at room temperature overnight in polyclonal rabbit serum primary antibody (anti-Sox10, kind gift of Bruce Appel) diluted 1 in 10,000 in block. Embryos were washed in PB-Triton once fast and three times for one hour. They were incubated overnight at room temperature in Alexa Fluor 488 fluorescent donkey anti-rabbit secondary antibody (Invitrogen, A21206) diluted 1 in 2000 in block. Embryos were then washed once fast and three times for 30 minutes in PB-Triton. They were placed in 50 % (v/v) glycerol for visualisation and storage.

### 2.3.2 In Situ Hybridisation

#### Probe Synthesis

20-40 µg DNA was linearised by cutting at the 3' end of the sequence to be used as a probe. Digestion was carried out in 200 µl for 2 hours at 37 °C with approximately 70 units of the appropriate enzyme. 5 µl of the reaction was run on a 1 % (v/v) agarose gel (Invitrogen) to check for complete digestion. The DNA was purified by phenol chloroform extraction and ethanol precipitation (see below). 1 µg template DNA, 2 µl NTP labelling mixture, 2 µl transcription buffer, 1 µl RNase inhibitor, nuclease-free water to final volume of 18 µl and then 2 µl of the appropriate RNA polymerase (all Roche) were added to a sterile microcentrifuge tube in that order. The mixture was mixed, centrifuged briefly and incubated at 37 °C for 2 hours. To clean the RNA probe a MEGAclear kit (Ambion) was used (see 2.7.1). 2 µl of the recovered probe was run on a 1 % (v/v) agarose gel for 10 minutes at 100 V to check for its presence. The remainder had 100 µl of hybridisation mix (see Appendix) added and was placed at -20 °C for storage.

#### Preparation of Zebrafish Embryos

Embryos were fixed overnight in 4 % PFA/PBS at 4 °C. To dehydrate them, embryos were washed once in PBT (PBS with 0.5 % (v/v) Tween) for 5 minutes and twice for 5 minutes in 100 % methanol and then placed in methanol at -20 °C at least overnight.

#### Staining Day 1 – Probe Hybridisation

Embryos were rehydrated by washing twice for 5 minutes in PBT. Embryos were then subjected to Proteinase K (10 mg/ml stock, Roche) digestion for varying times depending on age. 24 hpf embryos were incubated in a 1/10000 dilution of Proteinase K for 8 minutes. Older embryos were incubated in a 1/1000 dilution of Proteinase K for 20-45 minutes for 27-48 hpf embryos or 45-60 minutes for 50-60 hpf embryos. Digestion was followed by a brief wash in PBT and refixing for 20 minutes in 4 % (v/v) PFA/PBS. Embryos were then washed twice in PBT for 5 minutes each, once for

5 minutes in MilliQ water and once for 5 minutes in PBT. Embryos were pre-hybridised in hybridisation mix for 1-3 hours in a 68 °C water bath. Embryos were hybridised overnight at 68 °C in 100 µl hybridisation mix with 1/100 dilution of probe.

## Staining Day 2 – Antibody Binding

Hybridisation mix with probe was removed to be kept at -20 °C and recycled. The embryos were washed quickly in hybridisation mix at 68 °C and then twice for 30 minutes in hybridisation mix at 68 °C, once for 10 minutes in 50 % hybridisation mix/50 % PBT at 68 °C and once for 5 minutes in PBT at room temperature. Embryos were incubated for 2-4 hours at room temperature in block (PBT with 5 % (v/v) sheep serum, 2 mg/ml BSA). They were incubated overnight at 4 °C with anti-DIG alkaline phosphatase (Roche) diluted 1/2000 with block.

## Staining Day 3 – Colouration

The anti serum was removed. Embryos were washed once quickly in PBT, then six times for five minutes in PBT and three times for five minutes in NBT/BCIP buffer (see Appendix). Embryos were then incubated in staining solution in the dark at room temperature. For blue staining, 200 µl NBT/BCIP solution (Roche) was diluted in 10 ml of NBT/BCIP buffer. Reactions were stopped by washing quickly in PBT, 10 minutes in 100 % ethanol, briefly in PBT, 5 minutes in 0.1 M glycine pH 2.2 (Fisher Scientific) and again briefly in PBT. Embryos were then stored in 4 % (v/v) PFA/PBS at 4 °C. Before mounting for examination on a microscope embryos were transferred to 50 % (v/v) glycerol for at least 30 minutes.

### 2.3.3 LacZ Staining

Embryos were fixed in 4 % (v/v) PFA for between 30 and 60 minutes. They were then incubated overnight at 28.5 °C in staining solution (see Appendix) with XGal (5-Bromo-4-chloro-3-Indoyl-B-D-Galactopyranoside, Apollo Scientific) at a concentration of 1 in 40.

## 2.4 Microscopy

Stained fish were viewed whole mount by placing them in a drop of 100 % glycerol between stacks of No 1 coverslips on a slide, with a coverslip placed over the top. Live embryos were anaesthetised with Tricaine (0.002 % (v/v)) and mounted in this solution between stacks of No 1 coverslips on a slide, with a coverslip placed over the top. Embryos were viewed using a Nikon Eclipse E800 using either DIC or fluorescence microscopy as appropriate and photographed using a SPOT camera (Image Solutions) or Nikon sight DS-U1 camera (Nikon) together with NIS Elements F software or a

dual mode cooled CCD camera (Hamamatsu). For rapid sorting/scoring embryos were viewed on an MZ12 dissecting microscope (Leica).

## **2.5 Molecular Techniques**

### **2.5.1 Plasmid DNA Preparation for Cloning**

#### **Bacterial Growth Conditions**

Bacteria were either grown on LB agar plates or in LB media containing the appropriate antibiotic at the appropriate concentrations. These are: ampicillin at 50 µg/ml, kanamycin at 25 µg/ml or chloramphenicol at 27 µg/ml. Most cultures were grown overnight at 37 °C except for cultures with chloramphenicol which were grown at 30 °C.

Glycerol stocks of cells which had been transformed with a particular plasmid were made for ease of generating more DNA from those cells at a later date. 850 µl of overnight culture were vortexed together with 150 µl sterile 100 % glycerol and stored immediately at -80 °C. A small scraping of the frozen cells can then be used to seed new cultures.

#### **Midipreps**

Cells from a glycerol stock were picked directly into 50 ml LB medium with the appropriate antibiotic and grown overnight, shaking, at 37 °C. The cells were harvested the next day by centrifugation of 25-45 ml culture at 6,000 g for 15 minutes. DNA was prepared from the cells using a QIAfilter Midi kit (Qiagen). Pelleted cells were resuspended in 4 ml Buffer P1. 4 ml Buffer P2 were added and mixed by inverting the tube. This was incubated at room temperature for 5 minutes to lyse the cells. During this time a QIAfilter cartridge was prepared. 4 ml Buffer P3 was then added to the lysate and mixed by inverting the tube. This mixture was poured into the prepared QIAfilter cartridge and allowed to incubate for 10 minutes to allow the precipitate to separate. During this incubation a QIAGEN-tip 100 was prepared by applying 4 ml Buffer QBT to the tip and allowing the column to empty by gravity flow. Once the precipitate in the lysate had separated out a plunger was inserted into the cartridge and the cleared lysate expelled into the equilibrated QIAGEN-tip. The tip was allowed to empty by gravity flow and was then washed with two 10 ml washes of Buffer QC. The DNA was eluted in 5 ml Buffer QF. DNA was precipitated from the eluate by adding 3.5 ml isopropanol, mixing and centrifuging at 15,000 g for 30 minutes at 4 °C. The supernatant was decanted and the pellet washed with 2 ml 70 % (v/v) ethanol. The DNA was pelleted again with a 10 minute centrifugation at 15,000 g. Again the supernatant was decanted. The pellet was air dried and resuspended in 150 µl nuclease-free water.

## Minipreps

Colonies were picked from plates and were grown overnight in 3 ml LB media with appropriate antibiotic. Plasmid DNA was purified using Promega Wizard miniprep kits. 1.5 ml culture was harvested by centrifugation for 5 minutes at 14,000 rpm (Techne Genofuge 16M). The supernatant was discarded and the cells resuspended in 250 µl Cell Resuspension Solution. 250 µl Cell Lysis Solution were added and mixed, followed by 10 µl Alkaline Protease Solution. The lysate was incubated for 5 minutes at room temperature. 350 µl Neutralisation Solution were then added and mixed. The lysate was centrifuged for 10 minutes at 14,000 rpm. A spin column was inserted into a collection tube and after centrifugation the cleared lysate was applied to the column. This was centrifuged for one minute at 14,000 rpm. The flow-through was discarded and the column washed, firstly with 750 µl and then with 250 µl Wash Solution, by applying the solution to the column and centrifuging for one minute. The column was then centrifuged for a further 2 minutes to ensure all wash solution had been cleared from the column. The DNA was then eluted from the column by placing the column in a clean microcentrifuge tube, applying 100 µl nuclease-free water to the column and centrifuging for one minute. The DNA was checked on a 1 % (v/v) agarose gel before storage at -20 °C.

### 2.5.2 DNA Digestion

During cloning, plasmids were digested to obtain appropriate fragments for subsequent ligation into other vectors and to check for the presence of an insert after ligation and transformation had been carried out. Up to 10 µg plasmid DNA was digested in a volume of 200 µl with 3-5 µl of the required enzyme and 20 µl of the appropriate 10x buffer to obtain fragments for subsequent subcloning. Diagnostic digests were carried out on 4 µl miniprep DNA with 2 µl 10x buffer appropriate for the enzyme, 1 µl enzyme appropriate for the experiment and 13 µl MilliQ water. Most digests were incubated for 2 hours at 37 °C. The digests were then run on a 1 % (v/v) agarose gel to look for the presence of the expected bands. If the plasmid was not fully digested a further 1-3 µl enzyme were added and the incubation at 37 °C allowed to continue for a further 2 hours before checking again for complete digestion.

### 2.5.3 Oligo Annealing

Oligos to be annealed were diluted to 220 µM with MilliQ water. 4.5 µl of each oligo were mixed with 1 µl Annealing buffer (see Appendix). This mixture was placed at 65 °C for 10 minutes and then transferred to a beaker of water at 65 °C and allowed to cool slowly to room temperature. Annealed oligos could then be stored at -20 °C for later use in ligations.



### 2.5.4 Shrimp Alkaline Phosphatase (SAP) Treatment

SAP treatment was used to dephosphorylate DNA ends and prevent vector religation where cloning was non-directional. 1 pmol of 5' ends requires 0.1 units SAP (Promega). mol of ends is equal to the weight of DNA in the sample divided by the molecular mass of the DNA used, all multiplied by two. A 50 µl reaction was set up using Promega Buffer D and incubated at 37 °C for 90 minutes. The enzyme was heat inactivated at 65 °C for 15 minutes and the reaction stored in the freezer. The DNA was then purified before use (2.7.1).

### 2.5.5 Ligations

To subclone inserts, insert and vector were mixed in approximately a 3:1 ratio with 1 µl T4 ligase (Promega), enough buffer to produce 1x solution and MilliQ water up to 10 µl. The reaction was incubated at 4 °C overnight and then used to transform *E. coli* cells. For any particularly difficult cloning steps the ligation was microdialysed before transformation using nitrocellulose membranes (Millipore) over MilliQ water for 30 minutes.

### 2.5.6 Transformations

XL1-Blue cells were used for routine cloning and transformations, SCS110 cells were used to generate unmethylated DNA (both Stratagene). 0.85 µl beta mercaptoethanol (in kit with cells) was added to 50 µl of defrosted cells and the cells incubated on ice for 10 minutes. 1 µl plasmid DNA or 2 µl of ligation or all of a ligation that had been microdialysed was added to the cells and they were incubated on ice for a further 30 minutes. The cells were heatshocked at 42 °C for 45 seconds and then incubated on ice for 2 minutes. 950 µl preheated LB medium was added to the cells and they were incubated, shaking, at 37 °C for one hour. 50-250 µl of the transformed cells were spread on LB agar plates containing the appropriate antibiotic and were incubated overnight at 37 °C.

## 2.6 DNA/RNA Preparation

### 2.6.1 Genomic DNA Extraction from Fin Clips

Fish were anaesthetised in Tricaine (0.4 % (v/v) pH 7 diluted 4.2 ml in 100 ml of fish water). One half of their caudal fin was removed using scissors and placed in 100 µl of Genomic DNA extraction buffer (see Appendix). The fish were then returned to clean water and allowed to recover. Tail clips were incubated at 55 °C for 3 hours with occasional vortexing and then at 95 °C for 5 minutes to inactivate the Proteinase K. The DNA was centrifuged at 14,000 rpm for 20 minutes. 55 µl of the supernatant was diluted in 500 µl of MilliQ water of which 1 µl was used in PCR.

### **2.6.2 Genomic DNA Extraction from Whole Embryos**

Embryos were placed one per PCR tube and washed three times in MilliQ water. 25 µl of Embryo genomic DNA extraction buffer was added and tubes incubated at 55 °C for four hours and then at 95 °C for 5 minutes to inactivate the Proteinase K. 75 µl of MilliQ water was added and 2 µl of this solution used in PCR.

### **2.6.3 DNA Extraction from Bacterial Colonies for PCR**

Individual bacterial colonies were streaked onto fresh agar plates and grown overnight at 37 °C. Part of each streak was picked into 20 µl MilliQ water in PCR tubes and the samples boiled for 10 minutes to extract the DNA.

### **2.6.4 RNA Preparation using mMessage mMachine (Ambion)**

To generate sense RNA, plasmids were linearised by cutting at the 3' end of the insert and the DNA was phenol chloroform purified and ethanol precipitated (2.7.1). The transcription reaction was set up using mMessage mMachine kit in the following order: Nuclease free water to bring final volume to 20 µl, 10 µl 2x NTP/CAP, 2 µl 10x reaction buffer, 1 µg template DNA, 2 µl enzyme mix (SP6). The reaction mixture was mixed and incubated at 37 °C for 2 hours. 1 µl TURBO DNase was added, mixed and incubated at 37 °C for 15 minutes. RNA was recovered using a MEGAclean kit (Ambion, 2.7.3).

## **2.7 DNA/RNA Purification**

### **2.7.1 Phenol Chloroform Extraction and Ethanol Precipitation**

10 µl 3M NaAc and 110 µl phenol/chloroform/isoamylalcohol (25:24:1) per 100 µl of solution were added to the dirty DNA and vortexed briefly. The mixture was centrifuged at 14,000 rpm for 15 minutes. The top layer was collected and two volumes of 100 % ethanol added. This was stored at -20 °C overnight. The tube was centrifuged again at 14,000 rpm for 15 minutes. The supernatant was decanted, the pellet rinsed with 70 % (v/v) ethanol and left to dry at room temperature. The pellet was then resuspended in 20 µl of nuclease-free water. 4 µl of the resuspended DNA diluted in 96 µl MilliQ water was used to determine the concentration of DNA using a spectrophotometer.

### **2.7.2 Gel Extractions**

Where a fragment had been excised from a plasmid for subsequent subcloning the whole digest was run on a 1 % (v/v) agarose gel. The required band was then excised from the gel and the DNA purified from this band, using a QIAquick gel extraction kit (QIAGEN). When the band had been excised from the gel, the gel slice was weighed. Three volumes of Buffer QG were added to the gel slice in a 2 ml microcentrifuge

tube (i.e. 300 µl Buffer for every 100 mg of gel). This was incubated at 50 °C for 10 minutes until the gel slice had dissolved. One gel volume of isopropanol was then added to the sample and mixed. A QIAquick spin column was placed in a 2 ml microcentrifuge tube and the sample applied to the column. This was centrifuged for 1 minute at 14,000 rpm and the flow-through discarded. Where the volume of the sample exceeded 750 µl the column was spun once, then the remainder of the sample was added and the column spun again. The column was washed by applying 750 µl Buffer PE to the column, centrifuging for one minute at 14,000 rpm and discarding the flow-through. The column was centrifuged again at 14,000 rpm for one minute to remove all residual Buffer PE. The column was then placed in a clean microcentrifuge tube and the DNA eluted by applying 30 µl or 50 µl MilliQ water to the column, incubating at room temperature for one minute and centrifuging for one minute at 14,000 rpm. Recovery was evaluated on a 1 % (v/v) agarose gel.

### **2.7.3 RNA Purification using a MEGAclean Kit (Ambion)**

An RNA transcription reaction (from 2.3.2 or 2.6.4) was made up to 100 µl with Elution buffer. 350 µl Binding solution was added followed by 250 µl 100 % Ethanol. The mixture was mixed and applied to the filter in a collection tube. This was spun for one minute at 14,000 rpm. The collection tube was emptied and the filter washed twice with 500 µl Washing Solution. The filter was spun once more, for one minute, empty. To elute the RNA, 50 µl Elution Buffer preheated to 95 °C was applied to the filter and, with a clean collection tube, spun for one minute at 14,000 rpm. This was repeated to increase yield.

## **2.8 PCR**

### **2.8.1 Genotyping PCR Conditions**

PCRs were set up using 1 µl of CFP-F and CFP-R primers, the appropriate volume of DNA from fin clips or embryos, 10 µl GoTaq Green Master mix (Promega) and MilliQ water to a total volume of 20 µl. The PCR program used was: 94 °C 5 minutes, 35 cycles of 94 °C 30 seconds, 58 °C 30 seconds, 72 °C 1 minute, followed by 5 minutes at 72 °C and held at 10 °C. Products were run on a 1 % (v/v) agarose gel.

### **2.8.2 Colony PCR**

Colony PCR was used where cloning the correct insert into a vector was difficult. Using the technique large numbers of bacterial colonies from plates were screened in order to find the colony that contained the plasmid required. This avoided the need to do large numbers of time consuming and expensive minipreps and diagnostic digests. DNA was prepared from each colony as 2.6.3. PCR reactions were then set up using

10 µl DNA preparation, 2 µl primers, 12 µl BioMix or BioMix Red PCR mix (Bioline). The PCR program used was: 94 °C 3 minutes, 34 cycles of 94 °C 15 seconds, 55 °C 15 seconds, 72 °C 15 seconds, followed by 3 minutes at 72 °C and held at 10 °C. Products were run on a 1 % (v/v) agarose gel. PCRs using primers S21/S22 used the PCR program: 94 °C 5 minutes, 35 cycles of 94 °C 30 seconds, 58 °C 30 seconds, 72 °C 30 seconds, followed by 5 minutes at 72 °C and held at 10 °C.

## **2.9 Sequencing**

DNA was sent to MWG for sequencing ([www.mwg-biotech.com](http://www.mwg-biotech.com)). 1 µg DNA was air dried using a Speed Vac (Strattech Scientific). Non-standard primers were sent in addition to the sample in some cases, otherwise standard primers were provided by MWG. Sequence data was analysed using the BLAST tool on the NCBI website ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and Clone Manager SN software.

## **2.10 DNA/RNA Microinjection**

Embryos were injected using a Nanoject II (Drummond Scientific Co., Broomall, PA) at the one cell stage. Needles were pulled on a Micropipette puller (Sutter Instrument Co., Novato, CA) from 3 ½ ” Drummond glass capillaries (Drummond Scientific Co., Broomall, PA). DNA/RNAs were diluted in MilliQ water with 0.005 % (v/v) phenol red. Embryos to be processed for ISHs were fixed in 4 % (v/v) PFA overnight at 4 °C before being dechorionated, refixed at room temperature for approximately one hour and dehydrated according to the ISH protocol.

## **2.11 Promoter Analysis**

DNA sequence was submitted to TRANSFAC public version 6.0 using the Pattern Search for Transcription Factor Binding Sites (PATCH 1.0) interface. Parameters were set to look for vertebrate transcription factor binding sites of 6 bp or more with the maximum number of mismatches being set at zero (Matys et al. 2003).

## **2.12 Statistical Analysis**

Simple statistical analyses were performed in Microsoft Office Excel 2003. More complex analyses were performed in Prism 3.0.

# Chapter 3

## Pigment Mutants

### 3.1 Introduction

We discussed in 1.5.3 a model that we intend to test, adapted from one proposed by Kim et al. (2003), for the role of Sox10 in melanophores (Figure 1.04). One of the predictions from our model was that as well as promoting melanophore specification by activating expression of *mitfa*, Sox10 has a role to inhibit or delay melanophore differentiation by repressing, either directly or indirectly, the expression of genes downstream of Mitfa. In this Chapter we will test this prediction by exploring the zebrafish phenotypes of *sox10<sup>l3</sup>* and *mitfa<sup>w2</sup>* mutants. If Sox10 does repress expression of melanophore marker genes, then they will be derepressed in *sox10<sup>l3</sup>* mutants but remain repressed in *mitfa<sup>w2</sup>* mutants (which still express functional Sox10). This is a direct parallel to an experiment which revealed derepression of *Phox2a* in mouse *Sox10* mutants (Kim et al. 2003). We will also look at xanthophore marker gene expression to test the generality of our model in other neural crest derived pigment cells.

We know that *mitfa<sup>w2</sup>* homozygous embryos do not have any melanised cells (Lister et al. 1999) and embryos which are homozygous for the *sox10<sup>l3</sup>* mutation appear to lack all pigment cells too. However, closer observation reveals that *sox10<sup>l3</sup>* mutants have what are referred to as residual melanophores (Dutton et al. 2001). At 3 dpf, the residual melanophores appear as small specks of melanin distributed mainly in the dorsal stripe region. It is unclear whether these specks of melanin are melanophores whose morphology is abnormal or whether there is another explanation for them. Existing data shows that residual pigmented cells are not dependent upon expression of *mitfa*, since they are still present in *sox10<sup>l3</sup>:mitfa<sup>w2</sup>* double mutant embryos (Elworthy et al. 2003). Also, it has been previously reported that the melanophore marker *dct* is expressed in *sox10<sup>l3</sup>* mutant embryos at 24 hpf and 30 hpf (Kelsh, Schmid and Eisen 2000). This unexpected expression is seen in a premigratory neural crest position, just

where residual pigmented cells are located. These data are supportive of the prediction from our model that genes downstream of *Mitfa* will be derepressed in the absence of *Sox10* and that normally *Sox10* has a role to inhibit melanophore differentiation. To help explain these unexpected melanised cells in *sox10*<sup>3</sup> embryos, and to find out more about what they teach us about the role of *Sox10* in melanophores, we aim to examine them more closely. We will ask how early these residual cells first appear as well as observe their morphology and distribution. We would also like to discover how long *dct* expression persists and will also look for the expression of a wider set of melanophore marker genes in *sox10*<sup>3</sup> and *mitfa*<sup>w2</sup> embryos (where we would not expect to see residual expression) to confirm the pattern.

Melanophores are not the only non-skeletogenic cell type which appears to begin to differentiate in mutant embryos. Pigmented xanthophores have been observed in *sox10* mutant fish before 35-45 hpf but they are not morphologically normal and only appear in very low numbers (Dutton et al. 2001). Some iridophores, termed escapers, also appear in low numbers (typically 1-5) in a proportion of mutant fish. In contrast to melanophores and xanthophores, escaper iridophores appear entirely normal (Chapter 4 and personal communication, J. Müller). Iridophores, and the effects of *sox10* on their development, are being studied by others in the lab. However, we will look more closely at the development of xanthophores in *sox10*<sup>3</sup> embryos by looking for expression of early xanthophore markers. We predict that we may also see derepression of xanthophore markers in these embryos which would suggest that the model we are testing for the role of *Sox10* in melanophores may also be applicable in other neural crest derivatives.

The melanophore markers we have chosen to use are *dopachrome tautomerase (dct)*, *tyrosinase*, *silva* and *tyrosinase-related protein b (tyrp1b)*. *dct* encodes one of the enzymes involved in the later stages of the melanin synthesis pathway, and is well documented as an early melanophore marker (see Chapter 1). In zebrafish, it is expressed in melanoblasts from 19 hpf through to melanophores at the latest stage observed, 8 dpf (Kelsh et al. 2000). The three other markers we have chosen to use are often used in mouse melanocyte studies. *Tyrosinase (Tyr)* and *Tyrosinase-related-protein 1 (Tyrp1)* are two other tyrosinase family genes known to be involved in the melanin synthesis pathway and *Silver* is a gene encoding a structural melanosome protein (see Chapter 1 for details). In zebrafish, the *tyrosinase* expression pattern was documented by (Camp and Lardelli 2001). They saw expression of *tyrosinase* in cells in positions characteristic of early melanoblasts which persists until at least 46 hpf, when the pattern resembles that of melanised cells. Zebrafish have two copies of *tyrp1*, named *tyrp1a* and *tyrp1b* (Braasch, Scharl and Volf 2007). *tyrp1a* does not



have a documented expression pattern but *tyrp1b* does (Thisse et al. 2001). Between 19 hpf and 60 hpf it is expressed in cells in a pattern resembling that of melanoblasts/melanophores. Zebrafish also have two copies of the *Silver* gene, called *silva* and *silvb* (Schonthaler et al. 2005). *silvb* is expressed only in the pigmented retinal epithelium, *silva* is expressed here and also in cells resembling the pattern of melanophores at 2 dpf. Thisse et al. (2001) also report *silva* expression in the ‘neural crest’ between 16 hpf and 19 hpf and in ‘pigment cells’ between 19 hpf and 48 hpf; these authors did not identify the precise pigment cell type.

Xanthophores are a less well studied cell type and markers are therefore more difficult to come by. The two most regularly used markers are *gch* and *xdh*. Gch, GTP-cyclohydrolase I, and Xdh, xanthine dehydrogenase are both enzymes involved in the synthesis of pteridine pigments. These markers, however, come with a caveat that they appear to also mark melanoblasts at early stages (Parichy et al. 2000b). The product of Gch activity, tetrahydrobiopterin, is also important for the conversion of phenylalanine to tyrosine (a melanin precursor) and so the overlap seen with *gch* expression and lightly melanised cells (and therefore cells which are developing as melanophores) is perhaps not surprising. *xdh* expression was seen to overlap with *mitfa* expression (but not melanin). Parichy et al. (2000b) suggest these double positive cells may represent a population of melanophore/xanthophore precursors expressing genes indicative of both. It is of particular importance to bear this caveat in mind with reference to our work. We will not know the differentiation state of any of the cells which may be expressing either *gch* or *xdh* in *sox10*<sup>3</sup> mutants. Even at later stages we may be looking not at committed xanthoblasts/xanthophores, but at early stage melanoblasts or partially specified but as yet uncommitted xanthophore/melanophore precursors which, due to the loss of functional Sox10, have been unable to differentiate further. To attempt to make our results easier to interpret we have also used another, novel, xanthoblast marker. *paics* was identified as a xanthoblast marker both in a screen for novel pigment cell markers carried out in the lab (ERG and J. Müller, unpublished data), and by a microarray screen in progress in the lab (A. Boyd, T. Chipperfield, unpublished data). *paics* encodes an enzyme in the purine biosynthetic pathway (Li et al. 2007). Although we know that expression of *paics* does not overlap with melanin (T. Chipperfield, unpublished data), we cannot eliminate that it is not expressed together with *mitfa* in melanoblasts. A zebrafish *paics* transgenic insertion mutant exists which has a loss of xanthophore pigmentation, but whose neural crest derived melanophores appear normal (Amsterdam et al. 2004). This suggests that *paics* may never be expressed in melanoblasts/melanophores and that if it is, it does not perform an essential function in these cells. Use of *paics* as a xanthophore marker may provide some insight into xanthophore development in *sox10*<sup>3</sup> mutants but will be used with caution.



## 3.2 Results

### 3.2.1 Observations of Pigmented Cells in *sox10*<sup>l3</sup> Embryos

Residual melanophores in *sox10*<sup>l3</sup> mutants have previously been reported at 3 dpf but no mention was made of how much earlier they can be seen. Initially, we examined several embryos between 26 hpf and 72 hpf at low magnification. In these embryos, melanised cells were not seen at or before 31 hpf but were present at 39 hpf. From this timepoint the number of residual cells appeared to increase. We also had the impression that there were changes in the size and melanisation of these cells. Residual melanised cells are easily confused with other pigment cell types at low magnification. We ruled out that we were observing iridophores by also examining the embryos under incident light. We could still have been observing xanthophores, which sometimes take up methylene blue and become blue/green rather than yellow. We looked at another set of embryos at higher magnification to be sure we were not observing xanthophores and enabling us to document residual melanophores more comprehensively. We observed 29 embryos (Table 3.01) and scored the dorsal stripe in the region of each somite for whether or not there were melanised cells present. This can only represent an approximation of melanised cell number since more than one was often present in each somite.

Tables representing the data collected for each embryo can be seen in the Appendix and Table 3.02. From these tables, we can see that each embryo is unique in when and where melanised cells appear in the dorsal stripe. On no occasion were any melanised cells seen in *sox10*<sup>l3</sup> embryos at or before 35 hpf (24 embryos observed). One embryo at 36 hpf had melanised cells and two embryos at 37 hpf had melanised cells. In some embryos, development of residual melanised cells did not occur until even later, if at all. Thus, the time at which we begin to see residual melanised cells is variable between embryos but it does not occur before 36 hpf, representing a delay in melanisation of at least 12 hours in these mutant embryos compared to wild types.

Over a time course, the average number of somites per embryo which contain melanised cells increases (Table 3.03, Figure 3.01). When we examine the data tables for each embryo, we can see that this represents changes in the timing of the appearance of melanised cells within individual embryos rather than differences between embryos (Table 3.02). Although the embryos are highly variable, we can see that initially a small number of residual cells tend to be present in the most anterior somites. At later timepoints, as melanised cell number increases, the cells extend into more posterior somites. However, no melanised cells were seen beyond somite 24 and more usually they did not occur beyond somite 16.

Table 3.01 Times at which embryos were scored for residual melanised cells

Series	Number of embryos	Times observed, hpf					
		34	36	40	66		
1	19						
2	5	35	37	39	41	57	
3	5	39	41	43	45	58	

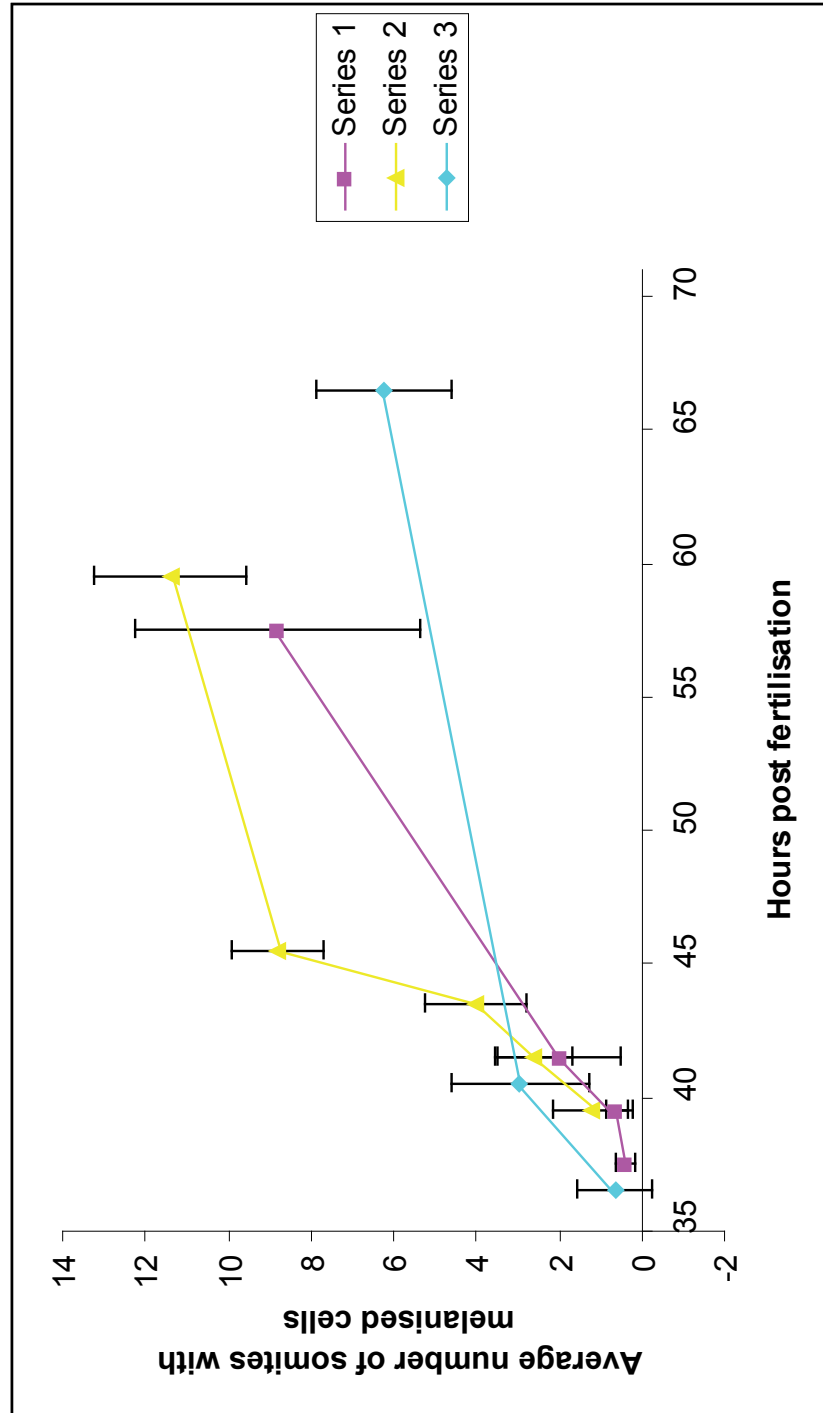
Table 3.02 The increase in the average number of somites containing melanised cells represents an actual increase in individual embryos

Hpfp	Somite 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25
39																									
41																									
43																									
45																									
59																									

Data for embryo 27 is depicted here as an example, each coloured square represents a somite with at least one melanised cell. The number of somites which contain melanised cells increases from none, to one, three, eight and then to 17. It is also possible to see that there is a spatial difference over time in the position of residual pigmented cells. Initially it is only the anterior somites which have residual cells. At later timepoints, somites which are more posterior also have residual cells. See also the Appendix.

Table 3.03 Average number of somites containing melanised cells increases over time (standard error in brackets)

Hpfp	34	35	36	37	39	40	41	43	45	57	59	66
Series 1	0 (0)		0.6 (0.9)			3.0 (1.7)						6.3 (1.7)
Series 2		0 (0)		0.4 (0.2)	0.6 (0.2)		2.0 (1.5)			8.8 (3.5)		
Series 3					1.2 (0.9)		2.6 (0.9)	4.0 (1.2)	8.8 (1.1)		11.4 (1.9)	



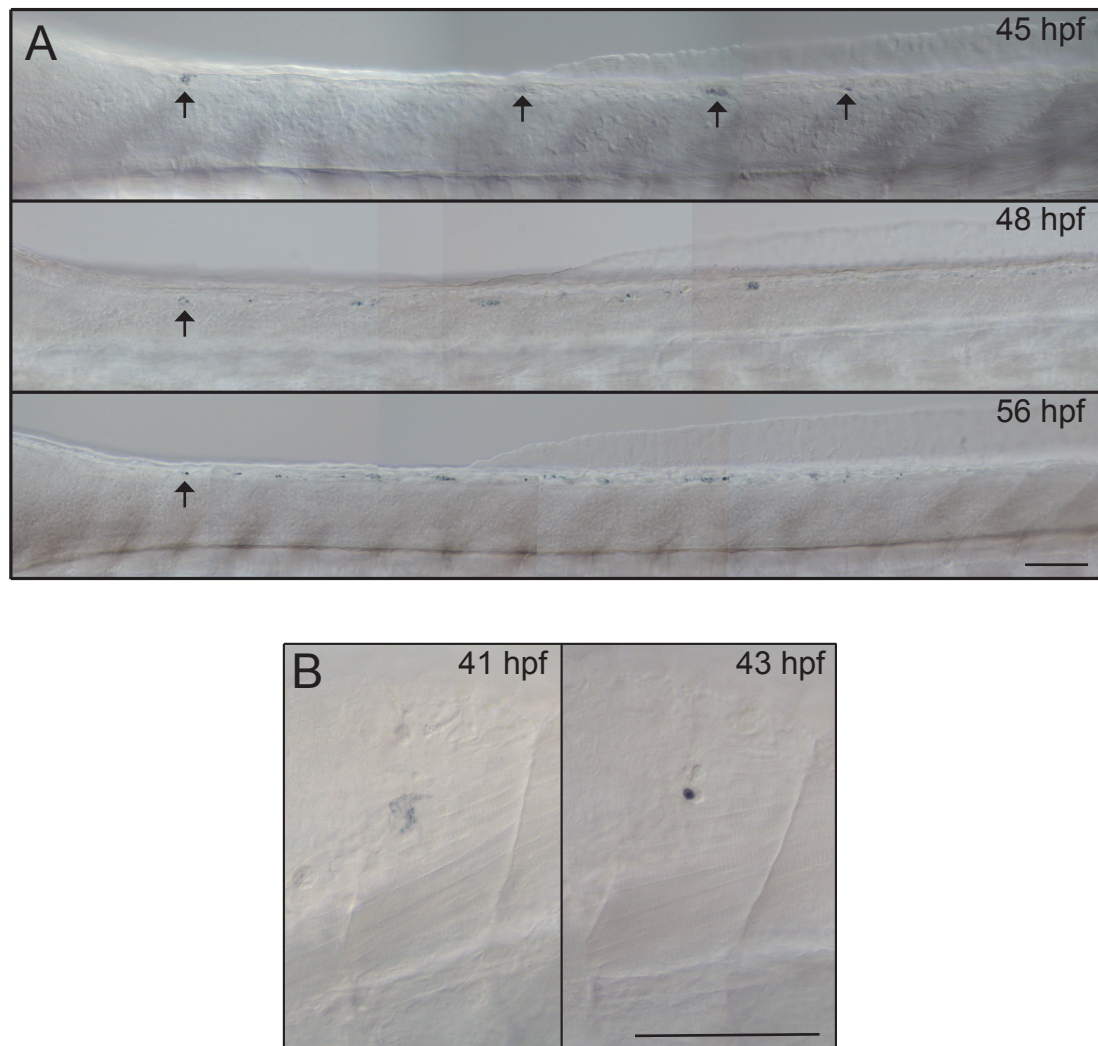
**Figure 3.01** The mean ( $\pm$  SE) number of somites with residual melanised cells increases over time  
Data is presented separately for Series 1-3, see Table 3.1 and 3.3.

We photographed a number of our embryos as we scored them, allowing us to compare individual somites every few hours. These photographs showed that the residual melanised cells are dynamic; they seem to change position and melanin distribution (Figure 3.02). One particularly striking example of this is shown in Figure 3.02. At 41 hpf we photographed a pigmented cell which was comparatively large and had a stellate morphology. By 43 hpf the cell appeared to have undergone melanin redistribution and could now be seen in the same somite, in the same position but it was much smaller, darker and rounded.

### 3.2.2 Melanophore Marker Gene Expression in *sox10*<sup>t3</sup> Embryos

We considered that the residual melanophores we had observed might represent the products of derepression of melanogenic genes. To investigate this possibility we carried out ISHs using melanophore marker genes in *sox10*<sup>t3</sup> mutant embryos. ISHs were carried out on embryos at six hour intervals between 24 hpf and 60 hpf from a *sox10*<sup>t3</sup> heterozygous incross. We used probes for *dct*, *silva*, *tyrosinase* and *tyrp1b* and each experiment was repeated on at least two occasions, with consistent results. Of the embryos in the figures for this section, embryos processed by ISH with probes for *dct*, *tyrosinase* and *silva* were all processed at the same time, although *silva* was allowed to develop for longer. The ISHs for *tyrp1b* were processed on a separate occasion but were carried out under comparable conditions. All ISHs were checked regularly and allowed to develop until no further staining became visible, plus a few hours longer. These ISHs had to be ‘pushed hard’ to allow us to see the expression in mutant embryos. Combined with the fact that we aimed to develop each stage for the same amount of time this means that some fish pictured may show more background staining than would be ideal. Where this is the case, it should be noted that on other occasions, when the same ISH was not allowed to develop for so long, the same pattern was always apparent but staining was weaker and there was no background staining.

As predicted by our model, all of the markers examined were expressed in *sox10*<sup>t3</sup> embryos. Mutant embryos were easy to differentiate from wild types by the pattern of expression in the neural crest. We could therefore score how many mutant embryos there were in a batch and how many of those displayed residual expression (Table 3.04). All of the markers used were expressed in the pigmented retinal epithelium in addition to the neural crest. This provided a useful means of knowing that the protocol had worked on individual embryos. No differences were seen between mutants and wild types in PRE expression and since we are interested in the neural crest we concentrate on expression in these cells. Where we state that there was no expression of a marker in an embryo, we exclude the expression seen in the PRE.



**Figure 3.02 Melanophore phenotype of *sox10<sup>3</sup>* mutants**

A. Over time, the residual melanophores in mutant fish become more numerous, darker and smaller. At 45 hpf in this example there are only four visible pigmented cells (arrows). These cells are small compared to wild type melanophores but larger than similar cells in later stage embryos. They are light in colour. By 48 hpf, the same fish has more pigmented cells. Some of the cells appear more punctate than others and are darker. At 56 hpf, there are still more pigmented cells and in addition, more cells appear punctate. Note how the most anterior cell at 45 hpf changes at 48 hpf and 54 hpf (arrows). Particularly comparing 48 hpf and 56 hpf, there is also an impression that some of the pigmented cells have moved or disappeared and been replaced by others.

B. Pigmented cell undergoing melanin redistribution. Over a timecourse it appeared that some residual cells first had a fairly normal morphology but then undergo melanin redistribution to form dark spots of pigment. Here we were able to capture a single pigmented cell either side of that event. At 41 hpf, the cell is large compared to other pigmented cells in *sox10<sup>3</sup>* mutants, though perhaps smaller than wild type equivalents, and has an uneven border, almost stellate as normal melanophores are. By 43 hpf the pigmented cell at the same location is smaller, darker and round. Scale bars 70  $\mu$ m.

Table 3.04 Number of embryos with residual expression of melanophore markers in *sox10<sup>l3</sup>* mutant embryos

	24 hpf	30 hpf	36 hpf	42 hpf	48 hpf	54 hpf	60 hpf
<i>dct</i>	7/7/26	6/6/24	5/6/25	6/6/28	5/5/24	1/3/24	0/4/23
	7/7/24	6/6/17	9/9/40	2/2/24	3/11/34	0/8/23	0/8/23
<i>silva</i>	6/6/20	4/4/25	6/6/23	8/8/28	8/8/26	4/8/24	0/6/25
	4/4/22	8/8/24	8/8/49	6/6/21	10/12/37	8/8/26	0/5/24
<i>tyrosinase</i>	3/3/23	5/5/24	4/5/25	6/7/23	0/6/23	0/5/24	0/2/23
	8/8/24	12/12/23	6/6/19	9/10/24	5/7/44	3/11/25	0/9/24
<i>tyrp1b</i>	7/7/24	8/8/25	5/5/22	7/7/24	2/2/24	0/1/23	0/4/23
	5/5/21		2/4/21		0/4/16		0/4/24

*sox10<sup>l3</sup>* heterozygotes were crossed and their progeny analysed by ISH using melanophore markers, *dct*, *silva*, *tyrosinase* and *tyrp1b* probes. The table shows the number of mutant embryos with residual expression followed by the total number of mutant embryos followed by the total number of embryos in each batch. If all of the mutant embryos showed residual expression of the markers the box is coloured blue, if none showed residual expression it is coloured yellow and if there was a mixture it is coloured green.

Table 3.05 Number of embryos with residual expression of melanophore markers in *mitfa<sup>w2</sup>* mutant embryos

	24 hpf	27 hpf	30 hpf	33 hpf	36 hpf
<i>dct</i>	0/1/23	1/3/22	2/6/24	3/4/24	0/4/25
	0/5/24	0/5/24	3/6/24	0/2/23	0/6/18
<i>silva</i>	2/4/18	5/8/22	1/3/21	3/7/25	0/4/16
	0/2/26	0/6/23	4/9/31	2/4/20	0/3/20
<i>tyrosinase</i>	4/4/20	7/7/24	4/4/19	1/2/22	0/3/22
	2/2/21	6/6/24	6/6/25	0/3/19	0/5/20
<i>tyrp1b</i>	2/3/18	0/3/22	0/6/24	0/8/22	0/5/25
	4/6/22	0/4/16	0/5/28	0/7/25	0/6/18

As for Table 3.04 except that *mitfa<sup>w2</sup>* heterozygotes were incrossed for this experiment.



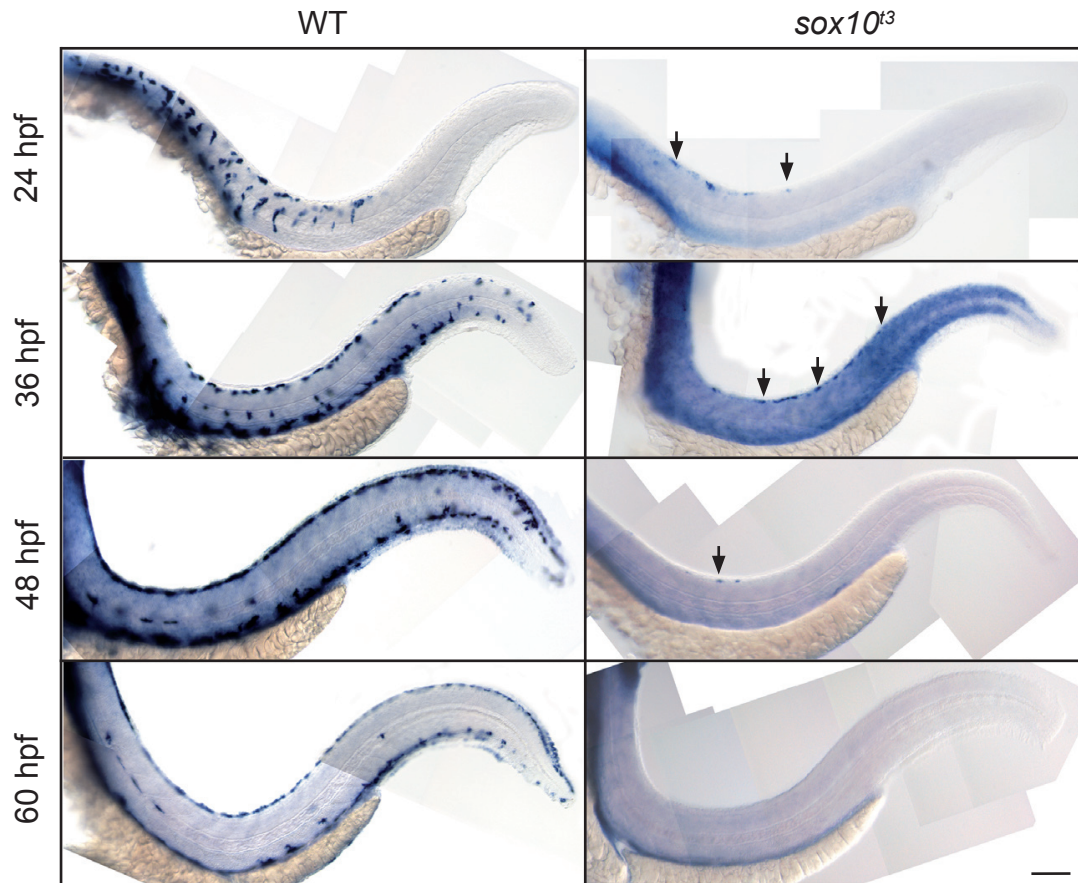
The pattern of expression for each of the markers was broadly similar. At earlier stages the markers were expressed in cells dorsal to the neural tube, the same area that the residual melanised cells are located. Within any given batch of mutant embryos there was often variation in the extent/strength of signal. At later stages there were progressively fewer cells expressing the markers, in progressively fewer mutant embryos until all mutant embryos showed no residual expression (Figures 3.03-3.06).

Despite the similarities, there are subtle differences between the patterns of expression with different markers. *dct* and *tyrosinase* have very similar patterns of expression in the mutant embryos both in terms of levels of expression (within the limitations of judging this by ISH) and in terms of spatial and temporal patterning of expression (Figure 3.03 and 3.05). Initially, at 24 hpf, there are low numbers of cells in a fairly anterior position in all of the mutant embryos. By 30 and 36 hpf, expression had extended more posteriorly. By 48 hpf and 54 hpf, the numbers of residual cells had noticeably decreased as had the occurrence of embryos with residual staining. At 60 hpf neither marker was expressed in mutant embryos. Loss of residual expression seems to be a gradual process which begins after approximately 36 hpf and ends in complete loss of marker gene expression at some time between 48 hpf and 60 hpf depending upon the embryo.

*silva* has a much stronger, broader and more persistent level of expression than any of the other melanophore markers (Figure 3.04). At 24 hpf, *silva* was expressed in a premigratory neural crest position but expressing cells were densely packed so that from a lateral view they appeared as a continuous line. Expression extended more posteriorly in the mutants than in the wild type embryos (well beyond the yolk sac extension) and more ventrally than the other markers in mutants at this stage. However, the trends in *silva* expression were much like that of *dct* and *tyrosinase*. At 36 hpf the area of cells expressing *silva* is much reduced, although distinct cells are still not easily distinguishable. By 48 hpf expression has reduced again such that individual cells can now be seen. As with the other markers, by this stage there is the occasional embryo which lacks any *silva* expression. At 54 hpf there are fewer embryos which express *silva* and those that do, do so in only a few cells. More embryos still have expression of *silva* at this stage when compared to embryos expressing *dct* at the same stage. At 60 hpf no embryos have any detectable *silva* expression.

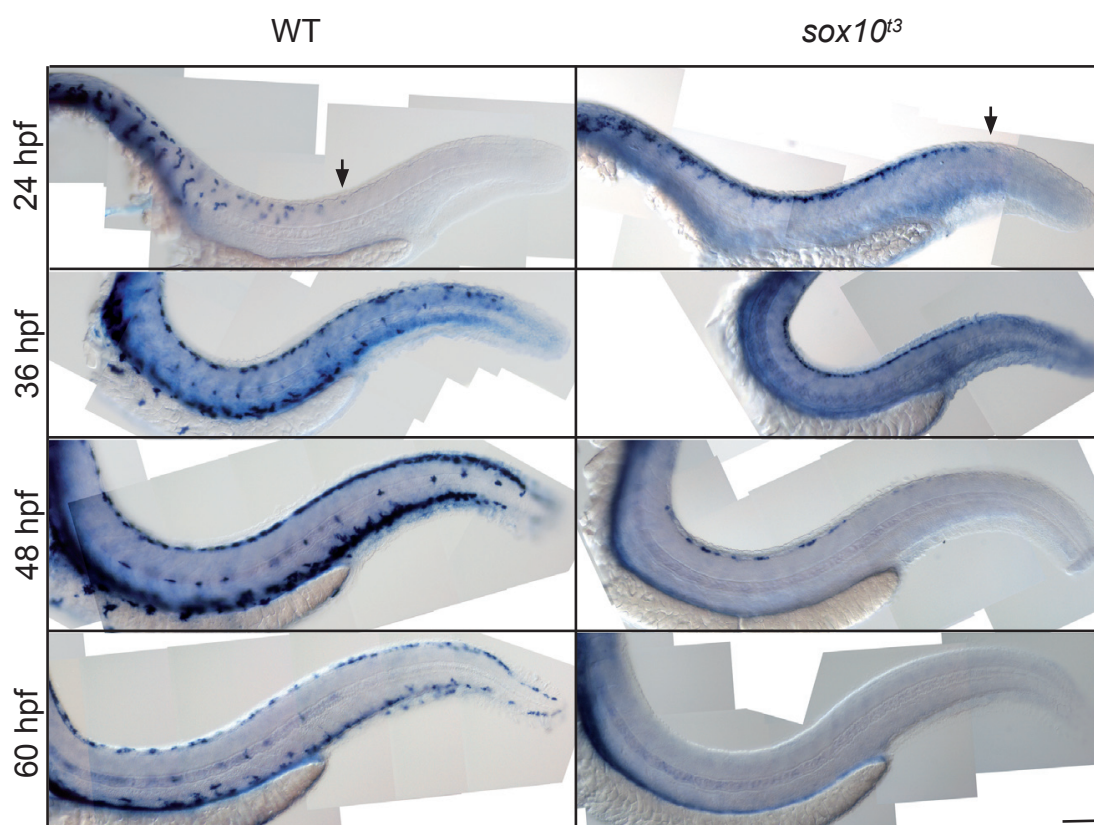
The temporal pattern of *tyrp1b* expression in *sox10*<sup>3</sup> mutants is similar to that seen for *dct* and *tyrosinase* (Figure 3.06). However, where a mutant embryo expresses *tyrp1b*, it does so only weakly and in very few cells; these cells are in a position that would be at the anterior end of the residual expression seen in the other markers.





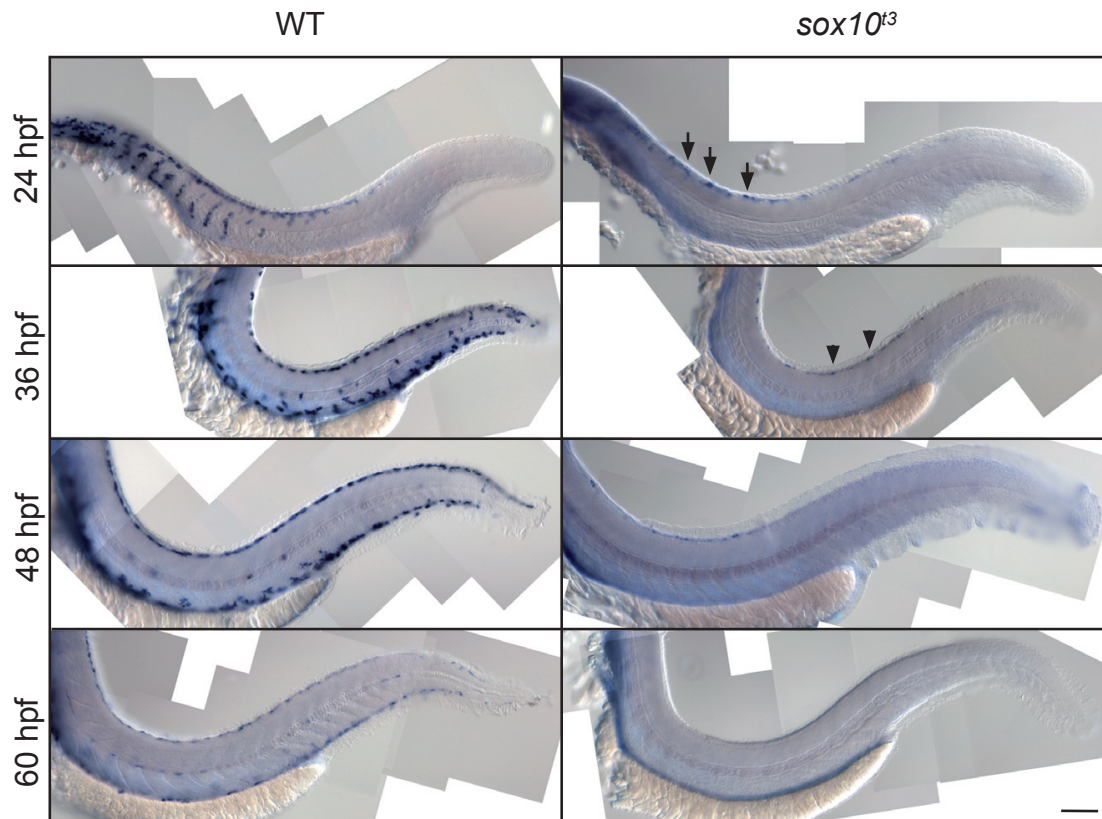
**Figure 3.03** *dct* expression in *sox10<sup>t3</sup>*

Wild type expression of *dct* correlates with the published expression patterns. Early, melanoblasts can be seen dorsal to the neural tube and migrating away along the medial and lateral pathways. Later, more cells can be seen migrating until by 48 hpf *dct* positive cells resemble the final pattern of differentiated melanophores in three stripes. In *sox10<sup>t3</sup>* embryos, where no melanophores properly differentiate, some residual *dct* expression is still seen. Cells are in a position dorsal to the neural tube (arrows) but are not seen in migratory positions. Expression is seen at 24 hpf in cells in the anterior trunk. Expression appears to extend more posteriorly at 36 hpf (note position of the most posterior arrows, marking the most posterior residual cells, in the figure at these timepoints). At 48 hpf there are fewer cells expressing *dct*, by 54 hpf and at later stages there is no detectable residual expression. Scale bar 100  $\mu$ m.



**Figure 3.04** *silva* expression in *sox10<sup>t3</sup>*

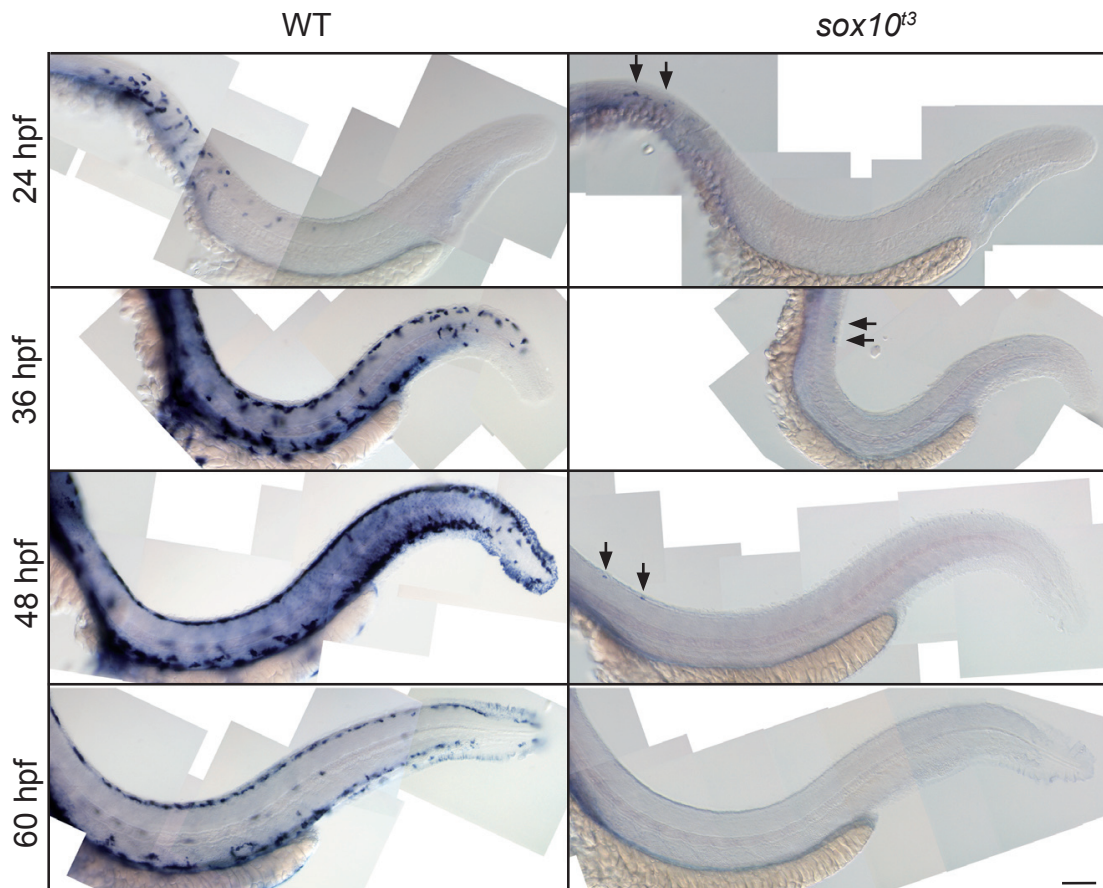
Wild type expression of *silva* correlates with published data, corresponding with the position of melanoblasts and later, melanophores. In *sox10<sup>t3</sup>* embryos, some residual *silva* expression is still seen in cells in a position dorsal to the neural tube but not in migratory positions. Strong expression is seen at 24 hpf in cells in the whole trunk and at this stage from a lateral view it appears as a solid streak of expression. Note also, that expression in the mutant embryos extends more posteriorly than in wildtypes (arrows), well beyond the yolk sac extension. At 36 hpf, expression levels are still high with expressing cells still forming a solid line dorsal to the neural tube but expression is weaker than at 24 hpf, particularly anteriorly. At 48 hpf there are fewer cells expressing *silva* so that we can now see individual cells, although in those cells expressing *silva*, the signal is still strong. By 60 hpf there is no residual expression seen in mutant embryos. Scale bar 100  $\mu$ m.



**Figure 3.05** *tyrosinase* expression in *sox10<sup>t3</sup>*

Wild type expression of *tyrosinase* correlates with the published expression patterns. In *sox10<sup>t3</sup>* embryos, some residual *tyrosinase* expression is still seen. Cells are in a position dorsal to the neural tube and are not seen in migratory positions. Expression is seen at 24 hpf in cells in the trunk, with a tendency for them to be concentrated more anteriorly (arrows). Expression at 36 hpf is slightly shifted posteriorly (arrowheads) and is generally weaker, appearing in fewer cells. At 48 hpf there are still fewer cells expressing *tyrosinase* with some embryos lacking expression altogether. By 60 hpf there is no detectable residual expression in any mutant embryos. Scale bar 100  $\mu\text{m}$ .





**Figure 3.06** *tyrp1b* expression in *sox10<sup>t3</sup>*

Wild type expression of *tyrp1b* correlates with the published expression pattern. The levels of *tyrp1b* expression in wild type embryos appears to be comparable to that of the other markers in wild type embryos. In *sox10<sup>t3</sup>* embryos, however, where no melanophores properly differentiate, residual *tyrp1* expression is still seen but only very weakly, compared to the other markers, and in a very few cells. Expression is seen in some embryos at 24 hpf, 36 hpf and 48 hpf in cells in the area just posterior to the ear (arrows). By 60 hpf there is no residual *tyrp1b* expression. Scale bar 100  $\mu$ m.

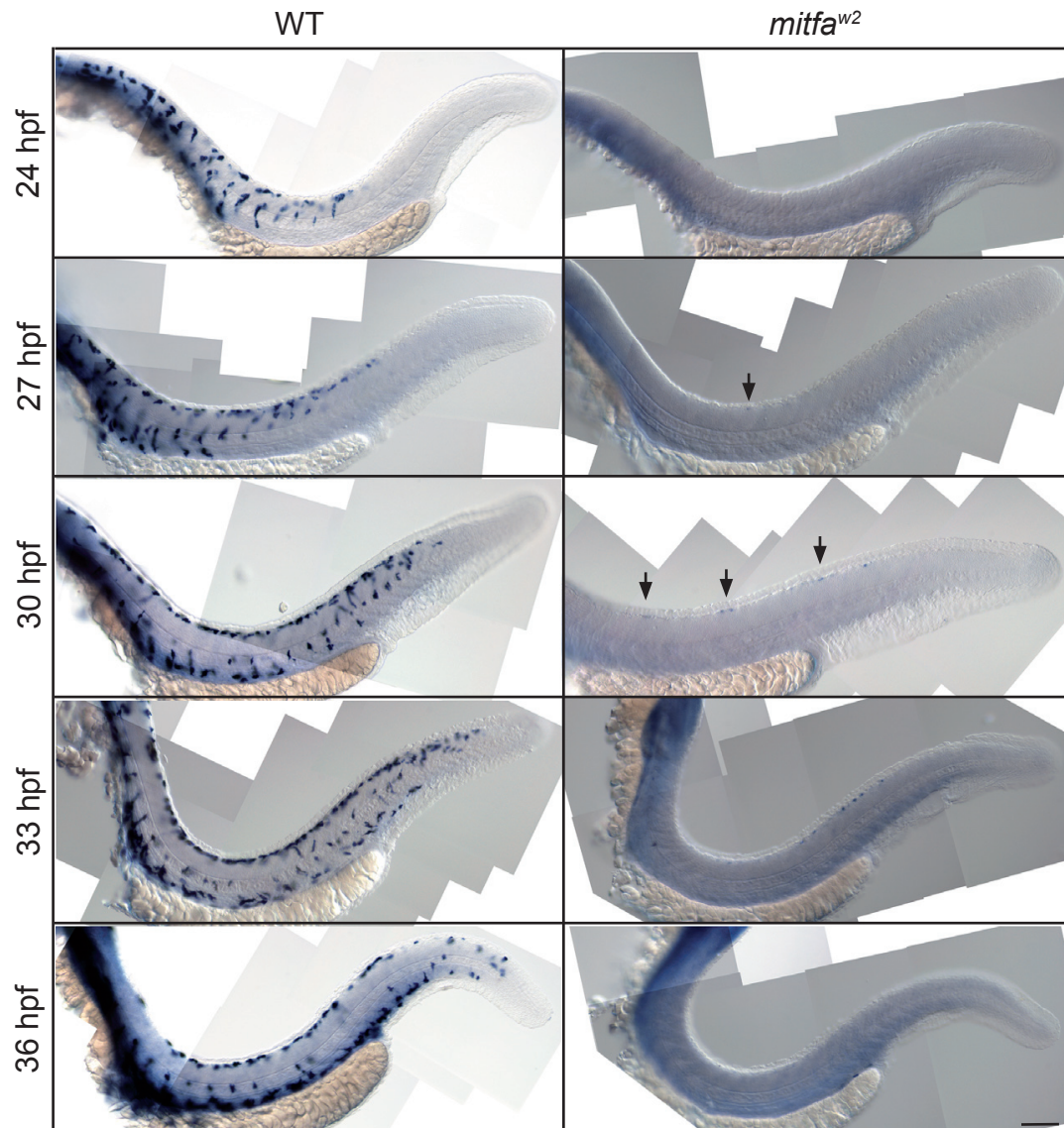
### 3.2.3 Melanophore Marker Gene Expression in *mitfa*<sup>w2</sup> Embryos

To determine whether the expression of our melanophore marker genes in *sox10*<sup>t3</sup> mutants was as a result of derepression by loss of Sox10, we also examined expression of our marker genes in embryos from a heterozygous *mitfa*<sup>w2</sup> incross. Our model predicts that there will be no expression of our marker genes in the neural crest of these embryos because Sox10 is still present to repress them. The embryos used in the figures in this section were processed and developed at the same time as their equivalents in the previous section allowing us to make as direct a comparison as possible between the two mutants. All of the markers are expressed at some time in *mitfa*<sup>w2</sup> embryos (Figure 3.07-3.11 and Table 3.05). Preliminary experiments showed that, in contrast to *sox10*<sup>t3</sup> mutants, expression is extremely weak and no expression of any marker is seen at, or after, 36 hpf (data not shown). As a result, we examined embryos over a shorter timecourse than for *sox10*<sup>t3</sup> mutants, every three hours between 24 hpf and 36 hpf.

Again, the four different markers share some similarities in their expression patterns in *mitfa*<sup>w2</sup> mutant embryos. All are present in cells in the premigratory neural crest position. Generally, expression is weaker and in fewer cells than in *sox10*<sup>t3</sup> mutants and at any given timepoint only some of the mutant embryos show any expression at all. The expression pattern of *tyrosinase* is most like that which we see in *sox10*<sup>t3</sup> mutants both in terms of positions of positive cells and strength of signal. The *tyrosinase* signal is generally strongest at 24 hpf and extends more posteriorly than we see at the same stage in *sox10*<sup>t3</sup> mutants. Expression then decreases both in strength and in numbers of cells, particularly from anterior positions, until at 36 hpf no *tyrosinase* expression is seen at all. Expression of *dct* and *silva* are slower to appear, being almost entirely absent from 24 hpf embryos, and are never seen in all embryos in a batch. Positive cells are located in the trunk and not in more anterior positions as in *sox10*<sup>t3</sup> mutants. The expression of *tryplb* is essentially absent from *mitfa*<sup>w2</sup> mutants. Only at 24 hpf did any embryos have *tryplb* expression, and then in only one or two weakly expressing cells in a very anterior position. These results support our hypothesis that the residual expression of melanophore marker genes in *sox10*<sup>t3</sup> mutants is as a result of loss of repression by Sox10.

### 3.2.4 Expression of Melanophore Marker Genes in *sox10*<sup>t3</sup>;*mitfa*<sup>w2</sup> Mutants

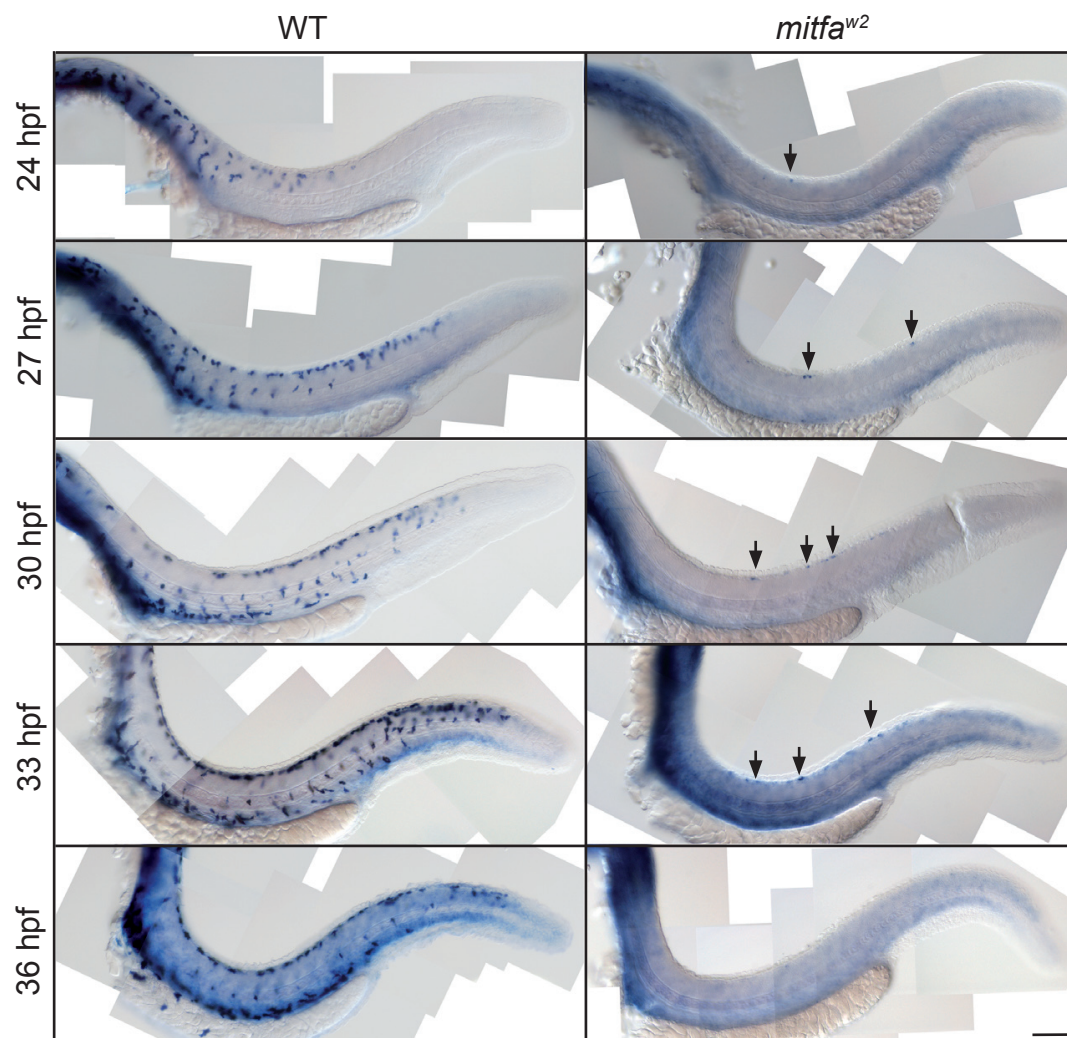
Embryos which were mutant for both *sox10* and *mitfa* also have residual pigmented cells (Elworthy et al. 2003). This indicates that the residual pigmented cells are not due to low level *mitfa* expression undetectable by ISH and supports the hypothesis



**Figure 3.07** *dct* expression in *mitfa*<sup>w2</sup>

Wild type expression of *dct* correlates with the published pattern. In *mitfa*<sup>w2</sup> embryos, where no melanophores differentiate, some residual *dct* expression is still seen, though only in a comparatively small proportion of embryos. Cells are in a position dorsal to the neural tube but are not seen in migratory positions. Expression is not seen at 24 hpf but by 27 hpf one embryo out of eight had a weakly expressing cell (pictured). Expression is greater, in terms of signal strength, number of cells and number of embryos with expression at 30 hpf. *dct* positive cells are located in the trunk of the embryo and do not extend as anteriorly as *dct* positive cells in *sox10*<sup>l3</sup> mutant embryos (arrows). This pattern persists at 33 hpf but expression is lost completely at 36 hpf. Scale bar 100  $\mu$ m.

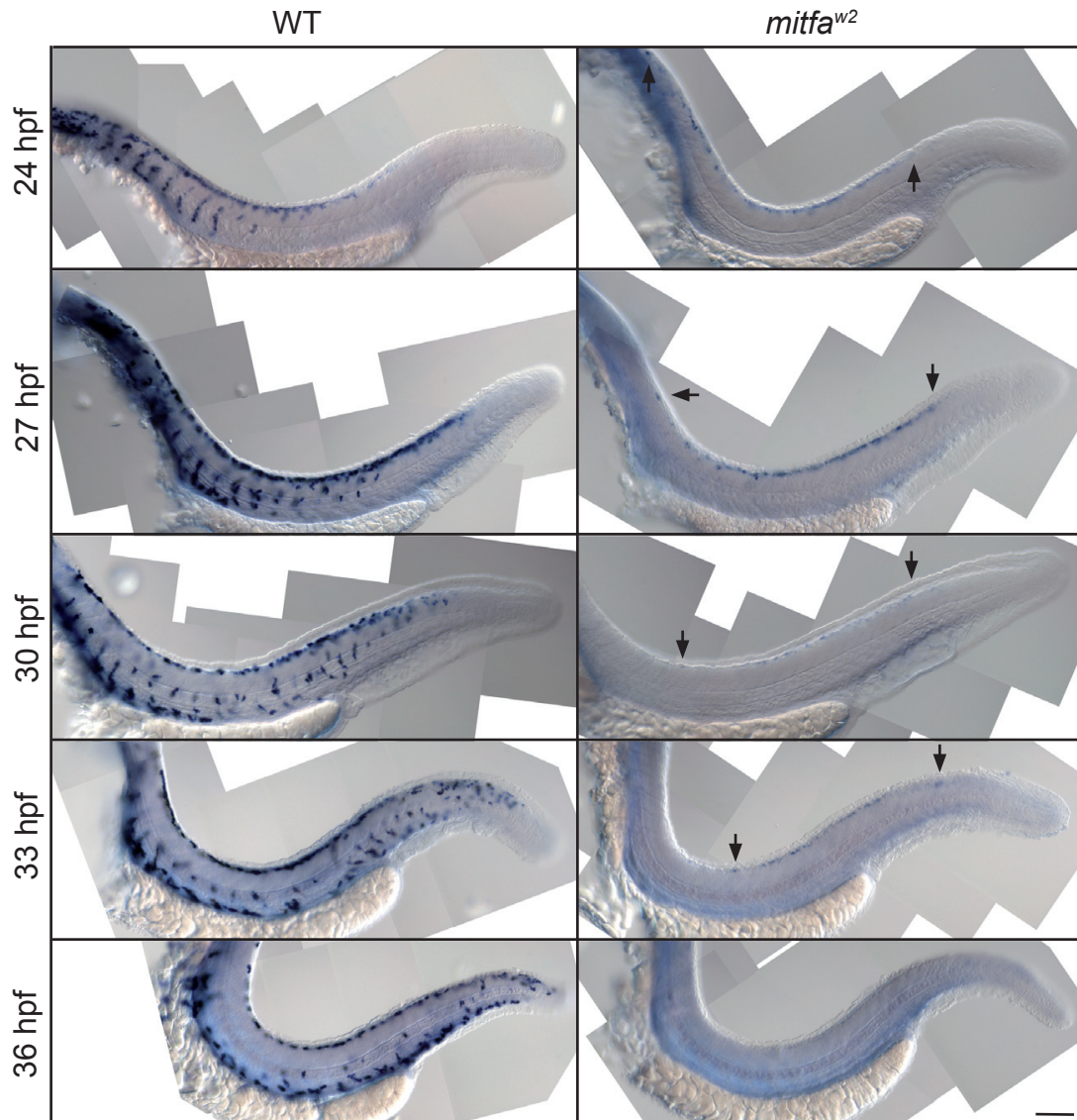




**Figure 3.08** *silva* expression in *mitfa*<sup>w2</sup>

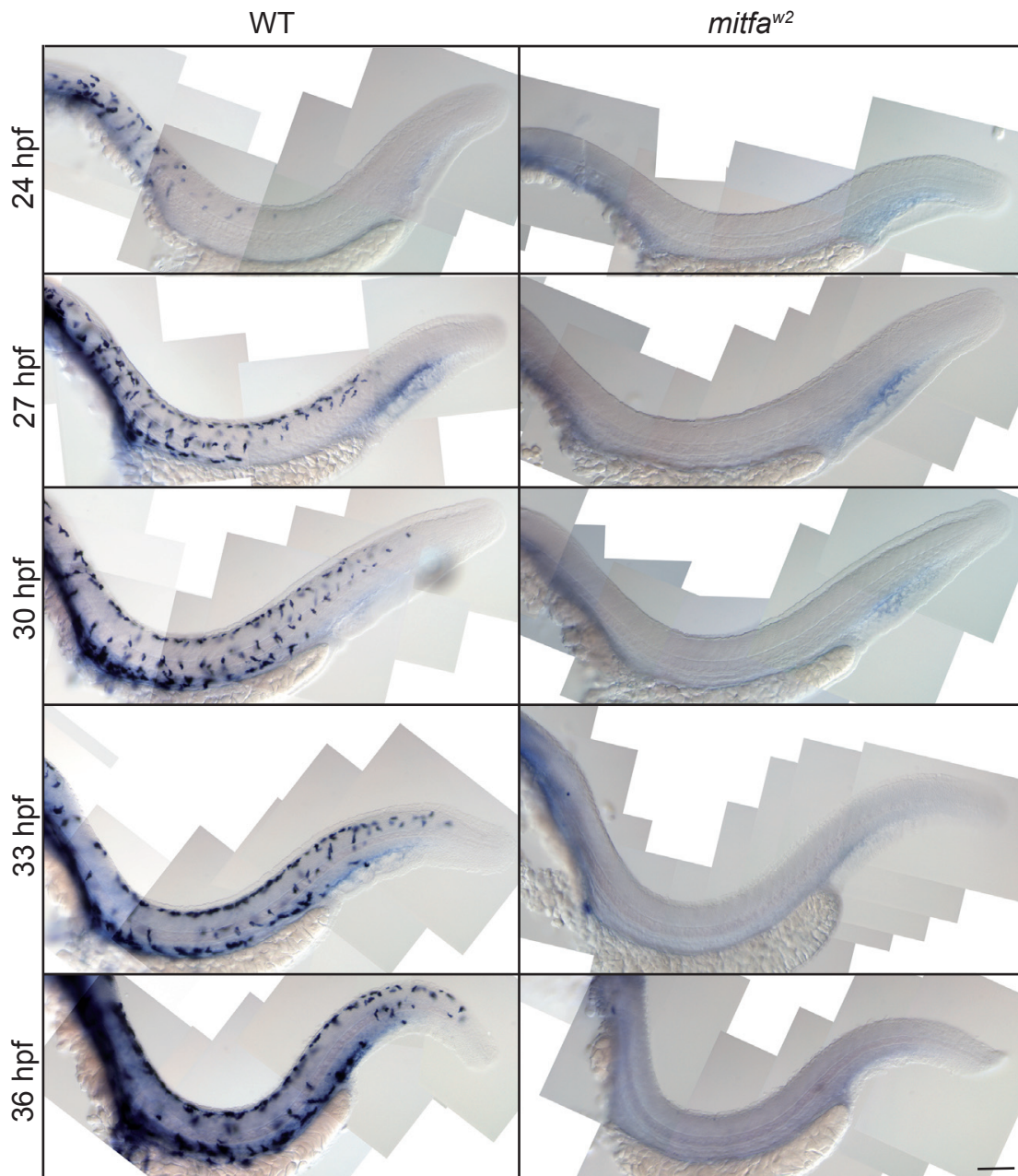
Wild type expression of *silva* correlates with published data. In *mitfa*<sup>w2</sup> embryos, some residual *silva* expression is still seen. Cells are in a position dorsal to the neural tube but are not seen in migratory positions. Expression is seen in about one third of the mutant embryos at 24 hpf in positions scattered along the trunk (arrows). Similar expression is seen at 27 hpf. Expression of *silva* is stronger than that of other markers in *mitfa*<sup>w2</sup> embryos but much weaker than *silva* expression in *sox10*<sup>3</sup> embryos. Expression persists at similar levels in similar positions until 33 hpf but is lost completely by 36 hpf. Scale bar 100  $\mu$ m.





**Figure 3.09** *tyrosinase* expression in *mitfa*<sup>w2</sup>

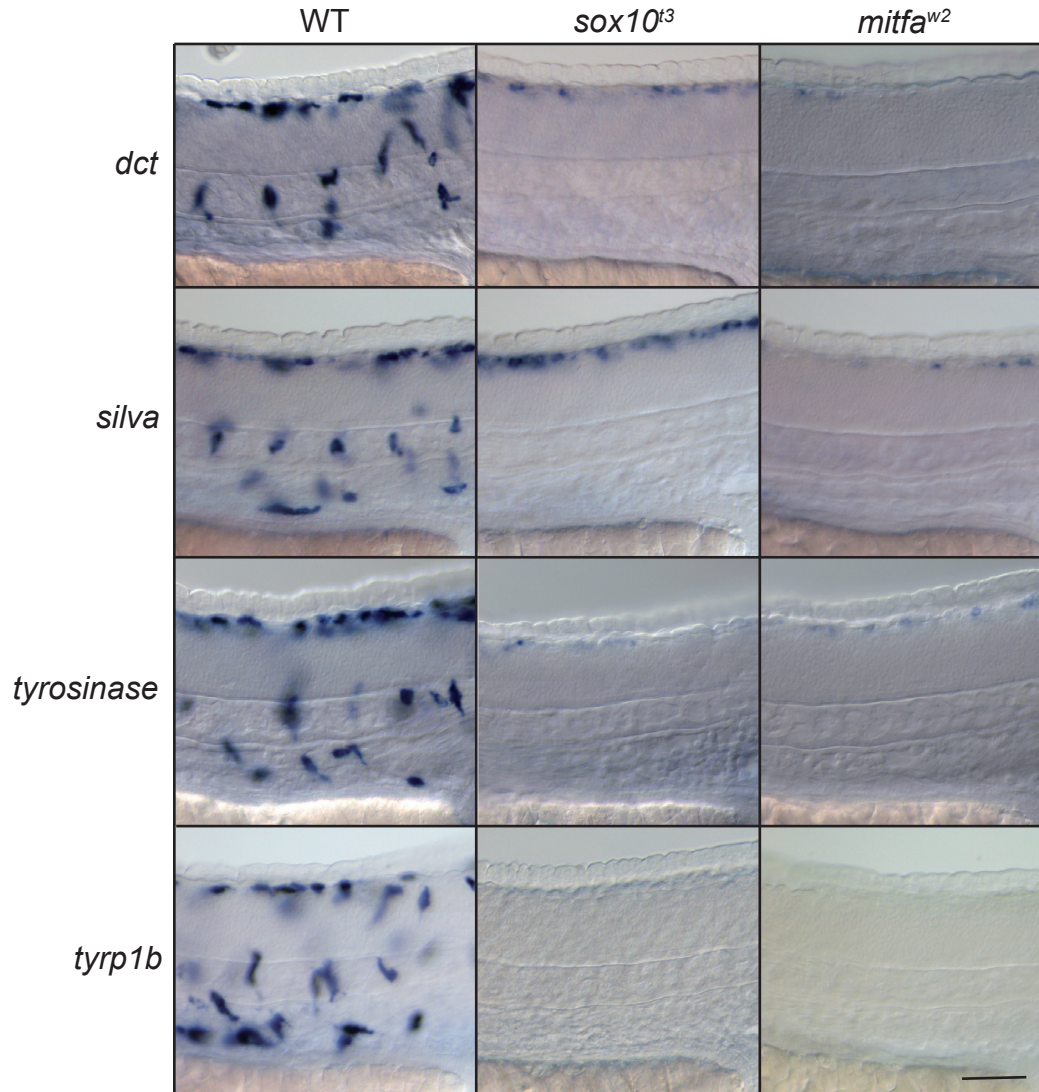
Wild type expression of *tyrosinase* correlates with the published expression pattern. In *mitfa*<sup>w2</sup> embryos some residual *tyrosinase* expression is still seen. Cells are in a position dorsal to the neural tube but are not seen in migratory positions. Expression is seen between 24 hpf and 33 hpf. Note the anterior and posterior limits of expression at each stage, the most posterior limit of expression changes little, whereas expression is lost in more anterior regions. By 36 hpf expression of *tyrosinase* is lost completely. Scale bar 100 μm.



**Figure 3.10** *tyrp1b* expression in *mitfa*<sup>w2</sup>

Wild type expression of *tyrp1b* correlates with the published data. In *mitfa*<sup>w2</sup> embryos, there are occasionally a couple of cells expressing *tyrp1b* at 24 hpf in very anterior positions (not shown) but no residual *tyrp1b* expression is ever seen at any other stage. Scale bar 100  $\mu$ m.





**Figure 3.11 Comparison of melanophore marker gene expression in wild type, *sox10<sup>t3</sup>* and *mitfa<sup>w2</sup>* 30 hpf embryos**

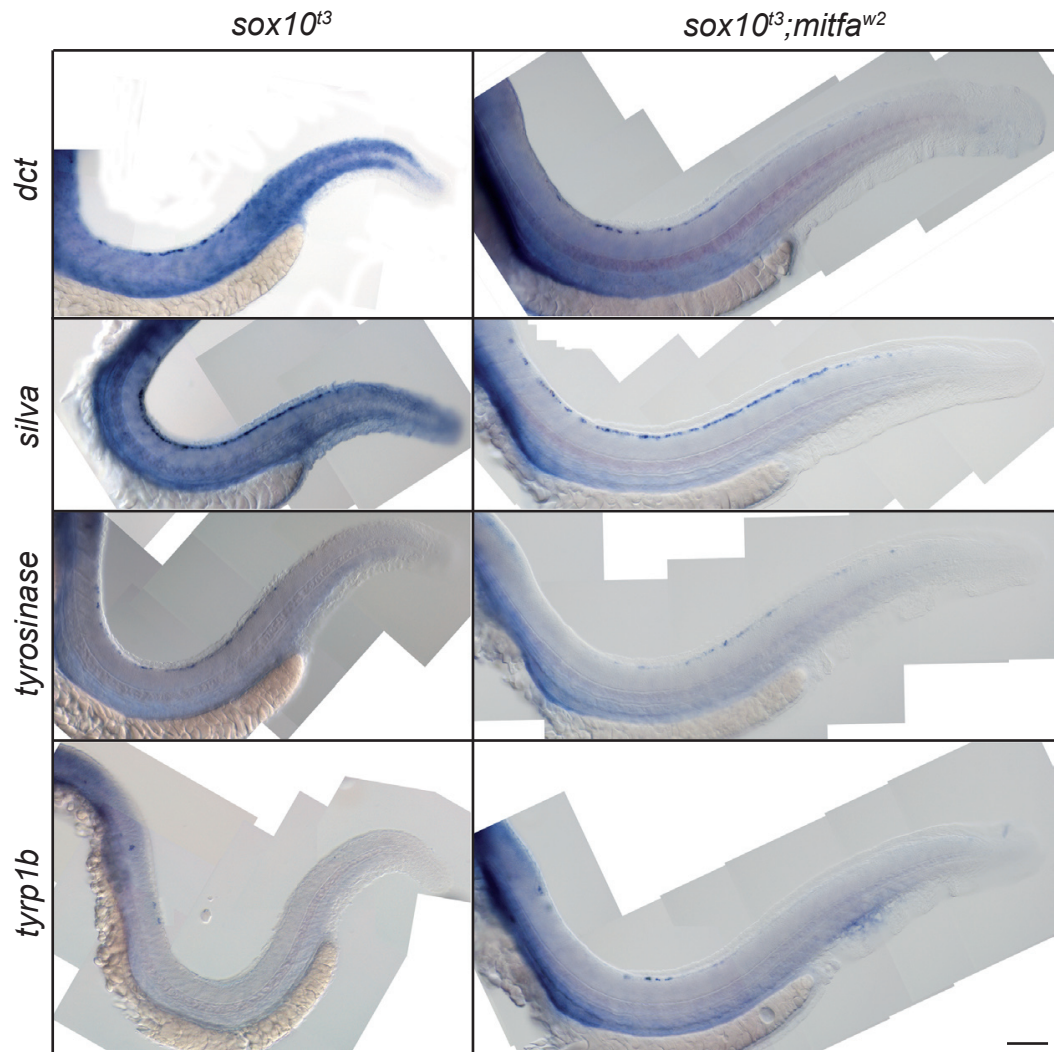
Images are of the region of the trunk anterior of the end of the yolk sac extension taken at a focal plane in the centre of the embryo so that the notocord is in focus. Expression of all markers is stronger in *sox10<sup>t3</sup>* than in *mitfa<sup>w2</sup>*. *silva* is the marker most strongly expressed in *sox10<sup>t3</sup>* and *mitfa<sup>w2</sup>*. *tyrp1b* expression is not visible in these images, its expression is confined to more anterior regions. Scale bar 50µm.

that they are due to derepression of melanophore differentiation genes. To further examine this hypothesis we generated fish which were homozygous for *mitfa*<sup>w2</sup> and heterozygous for *sox10*<sup>t3</sup>. Embryos generated from an incross of these fish were all homozygous for the *mitfa*<sup>w2</sup> mutation and one quarter were also homozygous for the *sox10*<sup>t3</sup> mutation and could be described as double mutants. If the additional loss of functional Sox10 on a *mitfa*<sup>w2</sup> mutant background has an effect, then one quarter of our embryos will have a different expression pattern than the rest. If not, then our double mutants will have the same marker gene expression patterns as *mitfa*<sup>w2</sup> single mutants and all of the embryos in these experiments will look identical. We carried out ISHs on embryos at 36 hpf, 48 hpf and 60 hpf for *dct*, *silva*, *tyrosinase* and *tyrp1b*. Two independent replicates of this experiment were carried out.

The most informative timepoint is 36 hpf (Figure 3.12 and Table 3.06). At this stage, there is robust residual staining of all of our markers in most *sox10*<sup>t3</sup> single mutants but none at all in *mitfa*<sup>w2</sup> single mutants. In our double mutants we saw approximately 25 % of the embryos had strong residual staining. We conclude that these embryos are double *sox10*<sup>t3</sup>;*mitfa*<sup>w2</sup> mutants. Ideally we would have liked to have genotyped these embryos to confirm which are double mutants but despite many attempts this proved impossible. However, supporting our conclusion, we also see expression of *dct*, *silva* and *tyrp1b* at 48 hpf and of *dct* and *silva* at 60 hpf in a few embryos, these are both stages when no expression at all is seen in *mitfa*<sup>w2</sup> single mutants and expression is being lost from *sox10*<sup>t3</sup> single mutants. This further supports the hypothesis that the normal function of Sox10 is to repress the expression of these genes, and melanophore differentiation, and that the residual expression we see in *sox10*<sup>t3</sup> mutants is *mitfa* independent.

### 3.2.5 Xanthophore Marker Gene Expression in *sox10*<sup>t3</sup> Embryos

We also examined expression of three xanthophore marker genes in *sox10*<sup>t3</sup> mutant embryos to see whether loss of Sox10 had similar effects upon different pigment cell types. Probes for *gch*, *xdh* and *paics* were used and each experiment was repeated on at least two occasions, with consistent results. ISHs were carried out on embryos from a *sox10*<sup>t3</sup> heterozygous incross. ISHs were processed and developed on separate occasions, but under comparable conditions. All ISHs were allowed to develop until no further staining became visible, plus a few hours longer. As with the ISHs with the melanophore marker genes, these ISHs had to be ‘pushed hard’, particularly at later stages and with the *xdh* probe, to allow us to see the residual expression in mutant embryos.



**Figure 3.12 Expression of melanophore marker genes in *sox10<sup>t3</sup>;mitfa<sup>w2</sup>* double mutants**

The pattern and strength of expression in the double mutants at 36 hpf is comparable to that seen in *sox10<sup>t3</sup>* single mutants. Scale bar 100  $\mu$ m.

Table 3.06 Marker gene expression  
in double mutants

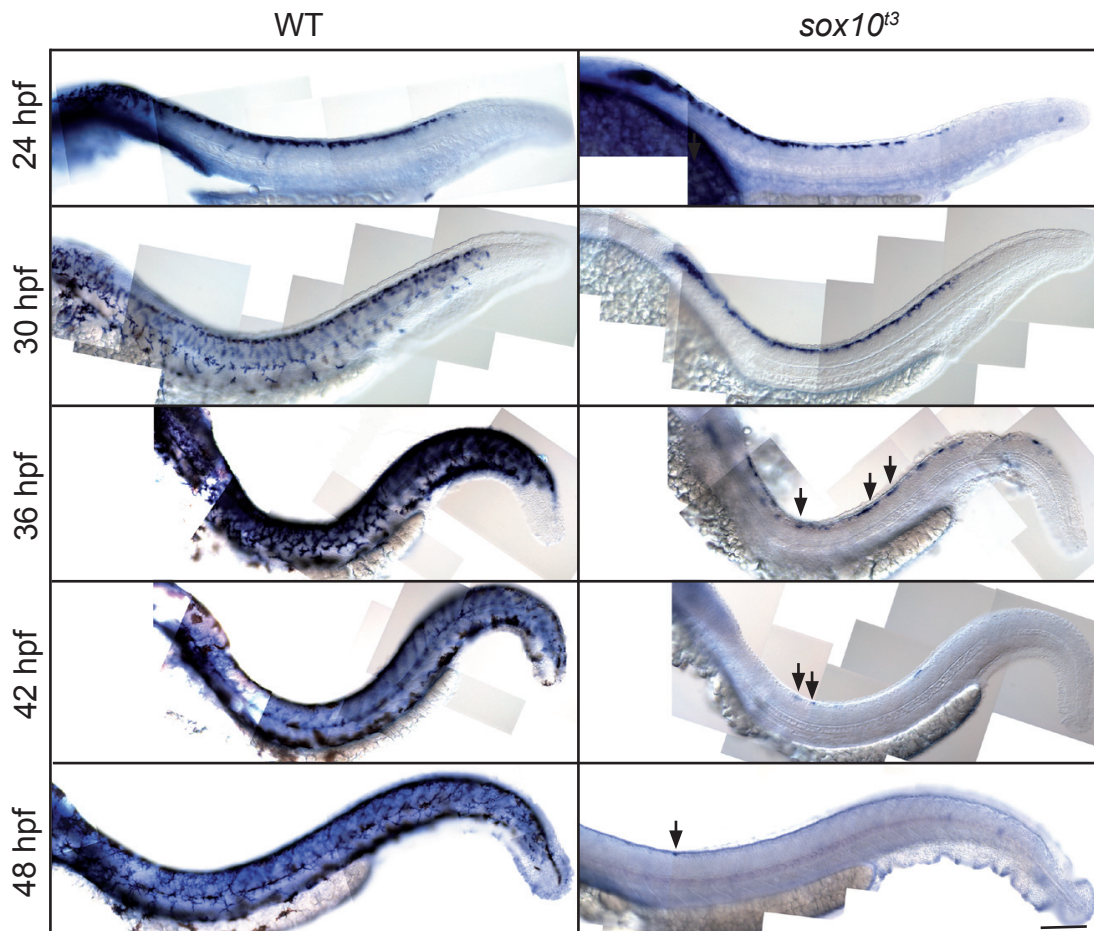
Age	<i>dct</i>	<i>silva</i>	<i>tyrosinase</i>	<i>tyrp1b</i>
36 hpf	2/24	3/22	5/24	2/19
	10/36	6/26	4/25	5/26
48 hpf	0/31	6/26	0/18	0/24
	6/17	5/27	6/23	4/25
60 hpf	0/29	0/28	0/25	0/23
	3/23	1/24	0/23	0/23

If expressed at all, all marker genes are expressed in approximately one quarter of embryos from an incross of fish which are homozygous for *mitfa*<sup>w2</sup> and heterozygous for *sox10*<sup>β3</sup>. We suggest that these fish are the double mutants in each batch. Where the marker genes are expressed in some embryos the table is coloured pink, where the marker genes are not expressed in any embryos the table is coloured yellow.

All of the markers examined were expressed in *sox10*<sup>l3</sup> embryos (Figure 3.13-3.16). Mutant embryos were usually easy to identify since the pattern of expression in the neural crest differed markedly from that in wild type embryos. Only *gch* presented difficulties at early stages where, due to the strength and broad pattern of expression, mutants and wild types were more difficult to differentiate. However, we were able to score how many mutant embryos there were in a batch and how many of those displayed residual expression (Table 3.07). All three xanthophore markers were seen to be expressed in premigratory crest positions and later only in cells migrating along the lateral pathway, a pattern indicative of xanthophores. The general pattern of expression of these markers in mutant embryos was very similar to that of the melanophore markers. All were expressed in cells in a premigratory neural crest position in mutant embryos. Overall, expression was broader/in more cells at earlier stages and later it was seen in fewer cells in fewer embryos until by 48 hpf all of the xanthophore markers are essentially absent from mutant embryos. For all of the markers, the ISHs took longer to develop in mutant as compared to wild type embryos during the development stage of the protocol, suggesting that expression was weaker in mutants as compared to wild types.

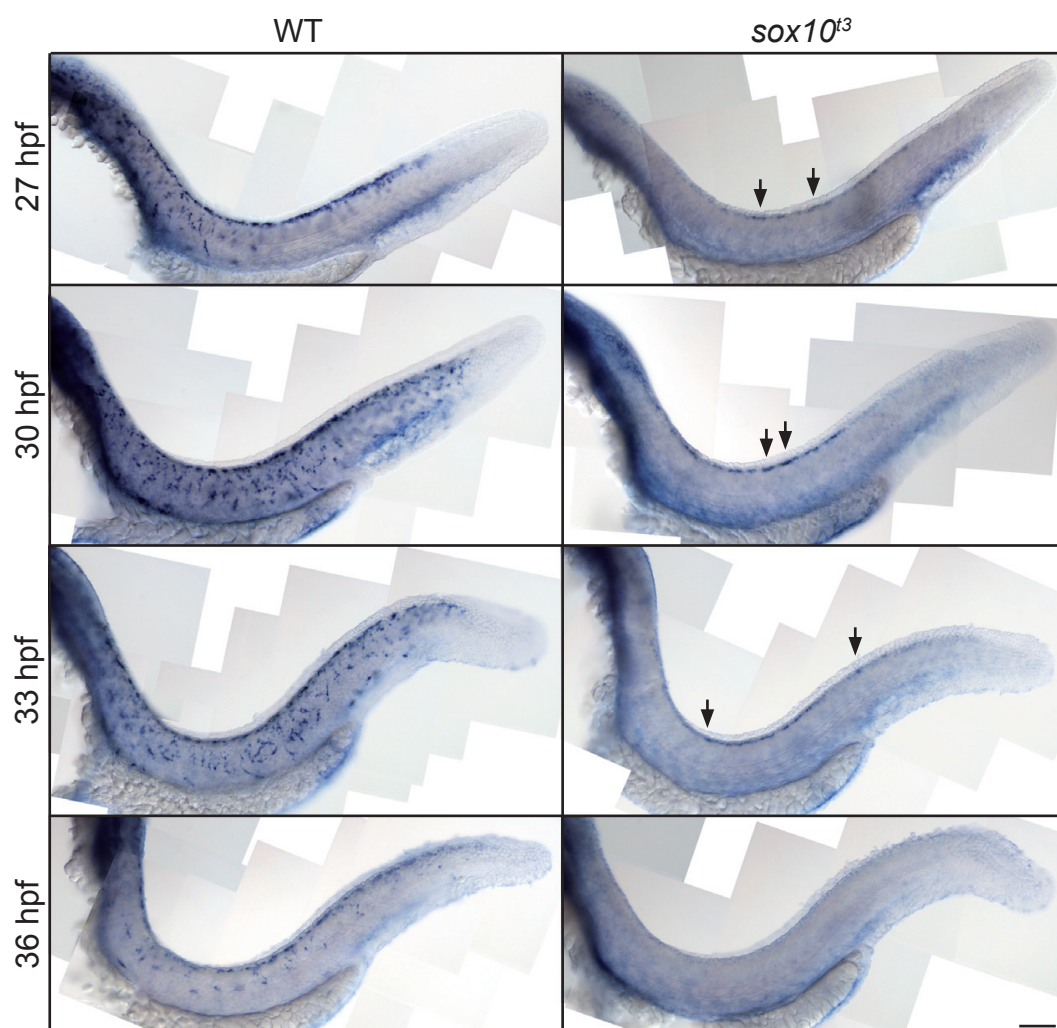
There were however, also differences between the markers. Particularly notable is the expression pattern for *xdh*. In accordance with the published pattern, the wild type expression of *xdh* is comparatively weak particularly at later stages although this is more pronounced in our figures which may be due to differences between the ISH protocol used in Parichy et al. (2000b) and our own. The timing of loss of *xdh* expression differs from the pattern seen with other markers; no expression is seen at stages after 33 hpf. This is much earlier than the other xanthophore markers which are still present at 42 hpf or any of the melanophore markers, which are not completely lost until 60 hpf. This may only reflect our inability to detect a very weak signal by ISH since our conditions do not seem to be optimal for this probe. At 24 hpf and 30 hpf expression of *gch* is broad and very strong. When we could identify mutants we did so by their lack of migrating cells. By 36 hpf, expression had decreased so that we could easily see individual cells. By 45 hpf and 48 hpf not all of the mutant embryos express *gch* and where an embryo does, it is only in very few (45 hpf), or one (48 hpf) cell. *paics* has an expression pattern that lies somewhere between those of *xdh* and *gch* and is most like that which we see for the melanophore markers. At 24 hpf, there are a few cells expressing *paics* in a fairly anterior position and by 30 hpf, expression has extended more posteriorly. By 36 hpf expression has begun to be lost again, being in fewer cells and in fewer embryos. We continue to see a loss of *paics* expression until at 48 hpf only a few embryos show expression in one cell.





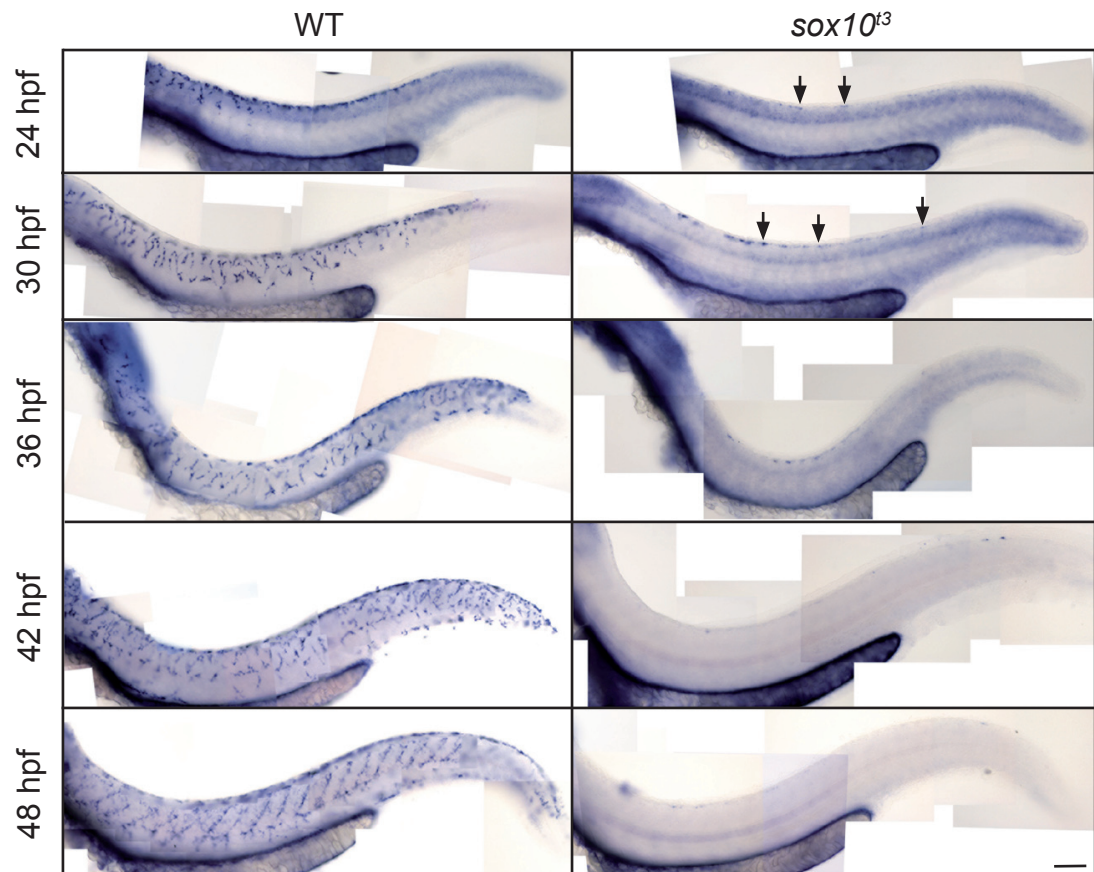
**Figure 3.13** *gch* expression in *sox10<sup>t3</sup>*

Wild type expression of *gch* is in stellate cells on the lateral pathway consistent with published data and with their being xanthoblasts and xanthophores. In *sox10<sup>t3</sup>* embryos, where xanthophores do not properly differentiate, residual *gch* expression is still seen. Cells are in a position dorsal to the neural tube but are not seen in migratory positions. Strong expression is seen in a dense line at 24 hpf and 30 hpf in very similar patterns dorsal to the neural tube. From this time, expression decreases, the number of cells expressing *gch* apparently becomes less so that we can see individual cells. At 48 hpf there is only the occasional cell in the occasional embryo still cell expressing *gch*.



**Figure 3.14** *xdh* expression in *sox10<sup>t3</sup>*

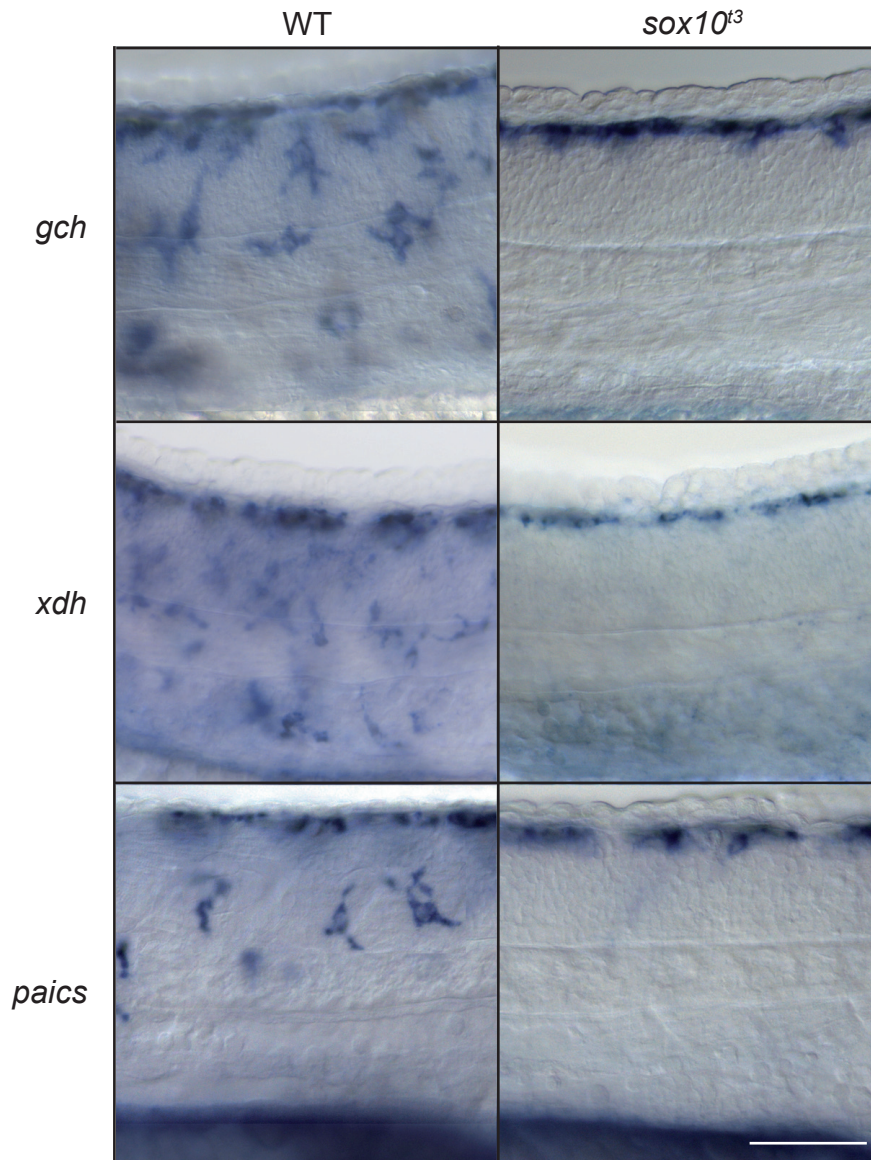
Wild type expression of *xdh* is, as published, in cells on the lateral pathway consistent with their being xanthoblasts/xanthophores. At 24 hpf, mutants and wild types are indistinguishable; cells can be seen dorsal to the neural tube and are more densely packed anteriorly. Later in wild type embryos, migrating cells can be seen. *xdh* positive cells always appear to have fairly weak expression. By 36 hpf, the wild type expression is very weak and in a decreased number of cells compared to 33 hpf. In *sox10<sup>t3</sup>* embryos, residual *xdh* expression is still seen. Cells are in a position dorsal to the neural tube but are not seen in migratory positions. Expression patterns at 27 hpf, 30 hpf and 33 hpf are very similar but by 33 hpf not all of the embryos are expressing *xdh*. At 36 hpf there are no residual cells still expressing *xdh*.



**Figure 3.15** *paics* expression in *sox10*<sup>3</sup>

Wild type expression of *paics* is in cells on the lateral pathway and not on the medial pathway, indicating that they are xanthoblasts/xanthophores. In *sox10*<sup>3</sup> embryos, some residual *paics* expression is still seen. Cells are in a position dorsal to the neural tube but are not seen in migratory positions. Expression is seen at 24 hpf in cells in the anterior trunk. Expression appears to extend more posteriorly at 30 hpf (note antero-posterior position of the arrows). By 36 hpf there are fewer cells expressing *paics* and by 48 hpf there is little or no residual expression. Scale bar 100  $\mu$ m.





**Figure 3.16 Comparison of xanthophore marker gene expression in wild type and *sox10*<sup>t3</sup> 30 hpf embryos**

Images are of the region of the trunk anterior of the end of the yolk sac extension taken at a focal plane in the centre of the embryo so that the notocord is in focus. Notice that *gch* expression in *sox10*<sup>t3</sup> embryos is strong and *xdh* expression is particularly weak. Scale bar 50  $\mu$ m.

Table 3.07 Number of embryos with residual xanthophore marker expression in *sox10*<sup>3</sup> mutant embryos

	24 hpf	27 hpf	30 hpf	33 hpf	36 hpf	39 hpf	42 hpf	45 hpf	48 hpf
<i>gch</i>	4/4/19		6/6/22				7/7/19		0/1/15
	19/19	11/11/51	2/2/15	5/5/23	2/2/17	11/11/24	6/6/21	7/9/22	4/12/24
<i>xdh</i>		7/7/23	6/6/25	7/9/25	0/4/16				
			3/3/19		0/4/19		0/7/23		0/11/24
<i>paics</i>	9/9/25		2/2/25		1/2/21		5/7/21		0/5/25
	6/6/25	6/6/24	8/8/23		7/7/21		4/5/24		2/6/23

The table shows the number of mutant embryos with residual expression followed by the total number of mutants embryos followed by the total number of embryos in each batch. If all of the mutant embryos showed residual expression of the markers the box is coloured blue, if none showed residual expression it is coloured yellow and if there was a mixture it is coloured green. On one occasion no mutants were seen and this box is coloured red.

### 3.3 Discussion

#### 3.3.1 Sox10 Represses Melanophore Differentiation Genes

*sox10*<sup>l3</sup> embryos have severe defects in a subset of neural crest cells including a dramatic reduction of all pigment cells, although residual melanised cells and escaper iridophores exist at 3 dpf in mutant fish and xanthophore-like cells are still seen before approximately 35 hpf. In Chapter 1 we proposed a model for the role of Sox10 in melanophores. One of the predictions from that model was that Sox10 promotes melanophore differentiation by activating expression of *mitfa*. Previous work by Elworthy et al. (2003) concluded that this was indeed the case. However, further to Elworthy et al. (2003) our model predicts that Sox10 also inhibits melanophore differentiation by repressing expression of melanophore differentiation genes. If this is the case then we would expect to see derepression of genes downstream of Mitfa in *sox10*<sup>l3</sup> mutants. Such derepression could explain the presence of residual melanised cells in *sox10*<sup>l3</sup> mutant embryos. We sought to look more closely at the phenotype of *sox10*<sup>l3</sup> mutants to test our prediction. *dct* has been previously described as being absent in *sox10* mutants at 21 hpf (when it is expressed in wild types) but present at 24 hpf and 30 hpf in cells dorsal to the neural tube (Kelsh et al. 2000). We wanted to find out if and when *dct* expression is lost from mutant embryos and to ask whether three other melanophore markers, *silva*, *tyrosinase* and *tyrp1b* are also expressed in *sox10*<sup>l3</sup> mutants. We found that all of our melanophore marker genes are expressed in *sox10*<sup>l3</sup> mutants from 24 hpf until approximately 54 hpf. We also looked for expression of the same genes in *mitfa*<sup>w2</sup> mutant embryos. If the expression of marker genes is reduced in those embryos which have functional Sox10 but lack Mitfa (*mitfa*<sup>w2</sup> embryos), as compared to those which lack both Sox10 and Mitfa (*sox10*<sup>l3</sup> embryos), this will support the hypothesis that Sox10 has a repressive effect upon the expression of melanophore marker genes. We found that expression of *tyrp1b* is almost entirely absent from *mitfa*<sup>w2</sup> embryos and that expression of the other marker genes occurs over a shorter time period, is much weaker and in fewer cells than in *sox10*<sup>l3</sup> embryos. We were also able to show that the strong residual expression seen in *sox10*<sup>l3</sup> mutants was not as a result of undetectable *mitfa* expression in these embryos since it is also observed in *sox10*<sup>l3</sup>;*mitfa*<sup>w2</sup> double mutants. Together, these data provide support for our hypothesis that Sox10 is able to inhibit melanophore differentiation by repressing expression of melanophore differentiation genes.

These data contrast with data about melanocyte marker gene expression in *Sox10* mutant mice. Of the four markers we have used in our work only *Dct* has been properly examined in mouse. It has been shown, both in vivo and in neural tube culture, that *Dct* is not expressed in the neural crest of Sox10 deficient mice (Britsch et al. 2001,

Potterf et al. 2001, Southard-Smith, Kos and Pavan 1998). However, it may be that *Dct* is expressed below the threshold at which it can be detected by ISH, we know that expression in zebrafish *sox10* mutants is very weak. The same authors also failed to observe pigmented cells in their experiments which may be an artefact of decreased sensitivity in detecting melanin in mouse as compared to zebrafish. Alternatively, it may suggest that there is no residual expression or that that which does exist is too little to generate melanin. It would be of interest to look at expression of *Tyrosinase* and *Tyrp1* in mouse *Sox10* mutants to confirm that these genes are not expressed either. If it was found that no melanophore specific genes are derepressed in *Sox10* mouse mutants it would suggest that there are significant interspecies differences between mouse and zebrafish in the way that melanophore development is regulated by Sox10 in each. Similar differences have been suggested before, by Hou et al. (2006), who demonstrated a late requirement for Sox10 to activate expression of *Tyrosinase*. There is no evidence for a late role for Sox10 in zebrafish melanophore development (Elworthy et al. 2003). Elworthy et al. (2003) showed that Sox10 directly activates expression of *mitfa* and conclude that subsequent expression of genes such as *dct* are not in direct response to Sox10 expression but that they respond via Mitfa. They excluded the possibility of a late requirement for Sox10 in melanophore gene expression in a series of overexpression experiments which demonstrated that Mitfa is able to rescue morphologically normal melanophores equally well in the presence or absence of functional Sox10. Further to this, and in contrast to data from Hou et al (2006) suggesting a positive late role for Sox10 in melanocyte development, our data suggest that Sox10 actually functions to repress expression of melanophore marker genes thus having a negative late role in melanophore development. These apparent interspecies differences will be discussed and explored further in later chapters.

### 3.3.2 Precocious Xanthophore Development in *sox10<sup>ts</sup>* Embryos

We also looked for residual xanthophore marker expression in *sox10<sup>ts</sup>* mutants to attempt to explain the residual xanthophores observed in *sox10<sup>ts</sup>* mutant embryos (Dutton et al. 2001, Kelsh and Eisen 2000) and to begin test the generality of our model in different pigment cell types. We found that our xanthophore markers were indeed expressed in similar spatial and temporal patterns as the melanophore marker genes in *sox10<sup>ts</sup>* mutants. *sox10<sup>ts</sup>* mutants display another phenotype reminiscent of the derepression phenotypes described for melanophore markers. When examined for fluorescence, *sox10<sup>ts</sup>* mutants can be reliably sorted from wild types at 24 hpf by the presence of cells which fluoresce in crest positions in the head and anterior trunk (55 out of 57 sorted embryos were mutant). The fluorescence persists at 27 hpf and 30 hpf. We know that our xanthophore markers are lost at later stages and that many neural



crest cells die between 35 hpf and 45 hpf (Dutton et al. 2001) so, as predicted, we found that by 54 hpf the fluorescence is gone. Xanthophores are autofluorescent and we suggest that what we were seeing was xanthophore autofluorescence some 11 hours prior to when it would normally be seen in wild type embryos. To confirm this we would need to photograph fluorescence in live embryos and process them by ISH for *gch*, *xdh* or *paics* for direct comparison. The precocious development of pigment in xanthophores is in contrast to the delayed development of pigment in melanophores and is probably due to the speed at which the necessary levels of enzymes required for pigment to form can be achieved in the different cell types. These data suggest that there is derepression of xanthophore marker genes in *sox10*<sup>3</sup> mutants and that our model may be more generally applicable in other neural crest derivatives. However, far less is known about xanthophore development than melanophore development. For example, we have no equivalent for *mitfa* in xanthophores, no master regulator of xanthophore differentiation. Without a xanthophore equivalent for the *mitfa*<sup>w2</sup> mutant it is difficult to confirm that the residual xanthophore marker gene expression we see in *sox10*<sup>3</sup> mutants represents derepression of those genes. Similarly, it is currently impossible to generate a genetic network to describe xanthophore development or a testable model for the role of Sox10 within that network.

### 3.3.3 Melanophore Development, Beyond Testing the Model

There are further implications for our understanding of melanophore development arising from the data presented here. A great deal of work in mouse and human has accumulated showing that Sox10 and Mitf can activate expression from the promoters of *Dct*, *Tyrp1* and *Tyrosinase* (Britsch et al. 2001, Potterf et al. 2001, Murisier and Beermann 2006, Murisier, Guichard and Beermann 2006, Murisier, Guichard and Beermann 2007, Bertolotto et al. 1998, Bentley, Eisen and Goding 1994). Both Sox10 and Mitfa are not functional/present in our mutants and yet some marker gene expression remains. However, we found that although expression of the markers appeared readily in wild type embryos, to see staining in the mutants we had to ‘push’ the ISHs hard at the developing stage. This indicates that gene expression in mutant cells is at comparatively lower levels than in wild type embryos. We also observed that residual expression of melanophore markers could be rather variable between mutant embryos in a single batch. Our results showing *dct* expression at 24 hpf and 30 hpf, recapitulated those in (Kelsh et al. 2000) and we also saw an anterior to posterior progression of *dct* positive cells similar to that which would occur in wild type embryos. The pattern for the other markers was comparable in this respect and counts of residual melanised cells also revealed an increase in their number over time together with an anterior to posterior progression. Whilst these pieces of evidence demonstrate the importance of Sox10 and Mitfa in the regulation of our melanophore marker genes,

either by direct or indirect regulation, it also indicates that there may be an additional mechanism by which these genes are activated. An attractive and plausible hypothesis is that the mechanism employed to achieve expression of melanophore marker genes in *sox10*<sup>3</sup> embryos is part of the mechanism by which those genes are activated in wild type embryos but which is less efficient in the absence of Sox10 and Mitfa. Genes of the Tyrosinase-related protein family from various organisms have binding sites in their promoters for Pax3 (Murisier and Beermann 2006) and Pax3 has been shown to regulate expression from the *Tyrp1* promoter in mouse (Galibert et al. 1999). One possibility is that Pax3 in a wild type embryo will act in conjunction with Mitfa to regulate expression of the *tyrosinase-related* family genes and that even in the absence of Mitfa it is still able to do this. Without Mitfa however, gene activation is inefficient which leads to variability in expression between embryos and explains why normal levels of expression are never achieved. This would be in contrast to data from adult mouse melanocyte stem cells where Pax3 has been shown to inhibit the expression of *Dct* (Lang et al. 2005).

This inefficiency of marker gene activation in the absence of Sox10 and Mitfa may also explain some of the observations we have made of residual melanised cells. We looked carefully at individual *sox10*<sup>3</sup> mutant embryos and did not observe any residual melanised cells, at or before 35 hpf but did find them from 36 hpf onwards. In wild type embryos melanisation begins at approximately 24 hpf so in *sox10*<sup>3</sup> mutants there is a delay in melanisation of at least 12 hours. There was also a great deal of variation in the time at which embryos gained melanised cells with some embryos failing to develop pigment at all. Activation of melanogenic enzymes appears to be inefficient in the absence of Sox10 and Mitfa so whether or not the genes are activated in particular cells is variable and it will take longer for the necessary components to accumulate resulting in the variability and delay in pigmentation that we see in *sox10*<sup>3</sup> mutants.

Expression of our melanophore marker genes in *sox10*<sup>3</sup> embryos is apparent from 24 hpf and progresses posteriorly until approximately 36 hpf. From this time, the number of positive cells decreases so that expression is lost entirely from some embryos as early as 48 hpf and from all by 60 hpf. This is consistent with data from Dutton et al. (2001) which showed that there is increased cell death in neural crest cells in *sox10*<sup>3</sup> mutants between 35 hpf and 45 hpf. They suggest that as a result of loss of Sox10, neural crest cells fail to specify and die by apoptosis. It is therefore likely that late loss of marker gene expression is as a result of apoptosis of expressing cells rather than loss of expression from cells which remain alive. However, we still see some embryos with some cells expressing our markers after 45 hpf, although there is great variability within each batch of embryos. Dutton et al. (2001) refer to 'a notable concentration of

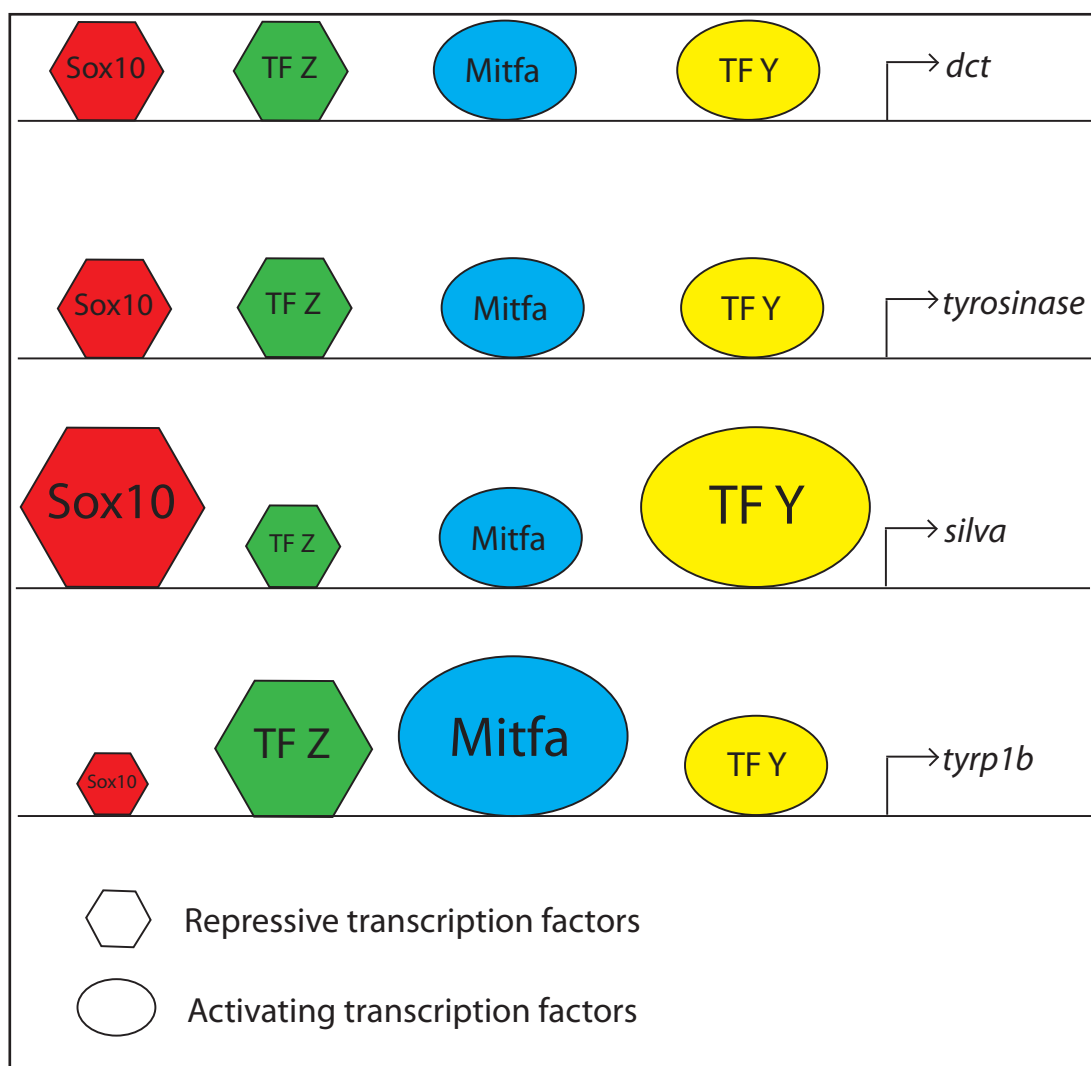
apoptotic cells' which they see between 35 hpf and 45 hpf but they do not detail what happens to the TUNEL staining after 45 hpf. From our results we would only expect a small number of cells to be dying after this timepoint and increased death of a small number of cells would have been difficult to detect by TUNEL staining. The data we have discussed so far enables us to conclude that melanophore development can continue to some degree even in the absence of Sox10 and Mitfa but that these cells are not able to develop normally and probably eventually die by apoptosis. Apoptosis is also the accepted fate of cells which fail to specify in mouse *Sox10* mutants (Britsch et al. 2001, Paratore et al. 2001, Sonnenberg-Riethmacher et al. 2001, Kapur 1999, Honoré, Aybar and Mayor 2003).

Most of the melanised cells that we observed in *sox10*<sup>3</sup> mutants were very small, round and darkly pigmented. We have considered whether these are the remains of melanised cells which have undergone apoptosis. We know that apoptosis is characterised by cell shrinkage followed by blebbing and the formation of apoptotic bodies (Lodish et al. 2001). We were able to capture images of a cell which appears to shrink and undergo melanin redistribution. At 41 hpf the cell in question was relatively large and of stellate morphology. At 43 hpf the same cell (judged by position and fortuitous lack of any other cells in the vicinity) was now much smaller, darker and round. Looking closely it also appears that there are other, unpigmented, bodies close by. We were only able to document this event once. This suggests that melanisation of neural crest cells in *sox10*<sup>3</sup> mutants is a rare event, because of inefficient gene activation, but that it does sometimes occur before apoptosis. By 3 dpf most of the residual cells observed are of similar apoptotic morphology, rounded, compact and darkly pigmented. To test whether residual melanised cells are apoptotic, we would need to carry out TUNEL staining on embryos between 36 hpf and 3 dpf and look for labelled, melanised cells.

Our observations of residual melanised cells in *sox10*<sup>3</sup> embryos illustrate again the failure of melanophores to form normally in these embryos. The residual melanised cells are fewer in number than melanophores in wild type embryos, are only seen in premigratory positions and do not look like wild type melanophores. This reiterates to us the importance of Sox10 and Mitfa for development of proper melanophores but begs the question why exactly do these cells not survive? Our observations so far have all been based upon melanin production; we have either looked at genes required for melanin/melanosome production or at melanin itself. Therefore, our results only indicate that Sox10 is not essential in directly or indirectly regulating genes involved in melanin synthesis. However, Sox10 or Mitfa may be an absolute requirement in the regulation of other genes needed for proper melanophore differentiation and survival. For example, a zebrafish c-Kit homologue, *kita*, is a transmembrane receptor present in

melanophores which is not important for melanophores to differentiate (*kita* mutants only have a reduced number of melanophores) but is important for their survival and migration (Parichy et al. 1999). It is known that *kita* is undetectable by ISH in *sox10*<sup>3</sup> mutant embryos (Dutton et al. 2001) and so it is likely that this gene requires either Sox10 or Mitfa to be expressed. *kita* is just one example of a melanophore survival gene that is completely absent from *sox10*<sup>3</sup> mutants and it is likely there are more, as yet undiscovered. For example, in mouse, *Bcl2* has been shown to be a melanocyte survival factor regulated by Mitf (McGill et al. 2002). This gene has been identified in zebrafish (Kratz et al. 2006) but little is known about its expression and function although we might expect similarities with mouse. We would predict that *Bcl2* will be expressed in zebrafish melanophores and that it will be regulated by Mitfa. Thus it may be another important survival factor missing from *sox10*<sup>3</sup> embryos.

Up until this point we have discussed the expression patterns of the melanophore markers in terms of their similarities and the general conclusions we can draw from those similarities. However, there are some points to note which are specific to each gene which might shed light upon more specific details of melanophore development. We described in the results section how similar, with respect to spatial and temporal patterning, the expression patterns of *dct* and *tyrosinase* are in *sox10*<sup>3</sup> mutants. These similarities suggest that perhaps the factors which regulate the expression of these two genes, beyond the requirement for Mitfa, are also similar. This might include regulation by Pax3 as discussed above but could also include regulation by other factors. *tyrp1b* positive cells are much lower in number and have weaker staining than any of the other markers. This implies that *tyrp1b* expression may not be repressed by Sox10 as much as the other genes. Alternatively, *tyrp1b* expression might be repressed by genes other than Sox10, expression of which are not affected by loss of functional Sox10, resulting in reduced residual expression. Another possibility is that expression of *tyrp1b* may be more dependent upon genes downstream of Sox10, i.e. Mitfa, than the other markers. Some of these concepts are illustrated in Figure 3.17. In contrast, whilst expression of *silva* follows the same temporal pattern of expression as either *dct*, *tyrosinase* or *tyrp1b*, spatially its expression is slightly different. *silva* is clearly expressed more broadly and in more cells than the other markers. This suggests that regulation of *silva* may be slightly different again from the other marker we have used. It may be that repression of *silva* expression by Sox10 is more important than for the other genes so that when functional Sox10 is lost, more cells are able to express *silva* than the other markers. Alternatively, it may be that there are factors which allow more efficient activation of *silva* in the absence of Sox10 and Mitfa compared to the other genes so that more cells express *silva* (Figure 3.17).



**Figure 3.17 Differential regulation of melanophore marker genes**

Repressor transcription factors are represented by hexagons and activator transcription factors by ellipses. TF Z and TF Y represent unknown, hypothetical transcription factors also involved in the regulation of melanophore marker gene expression. The size of each transcription factor represents its predicted importance for gene expression. From our results, the regulation of *dct* and *tyrosinase* appear to be similar. There is also no evidence that any one transcription factor is important over and above the rest. However, from our data it appears that Sox10 may particularly important for *silva* repression. Alternatively, or perhaps in addition, TF Y may be more important than Mitfa for *silva* expression. Sox10 seems to be less important for regulation of *tyrp1b* suggesting that Mitfa might be a key activating factor for this gene or that TF Z may be more significant for regulation of this gene than the others.

It would be interesting to know how far the expression of *silva* overlaps with that of the other marker genes. Which combinations of genes does a cell need to become melanised? Work from mice suggests that all of the genes we have examined will be required for melanin synthesis, since loss of any one results in changes in coat colour. Mouse *Tyrosinase* mutants have white coats, (Beermann, Orlow and Lamoreux 2004) and the zebrafish *tyrosinase* mutant (*sandy*) has unpigmented melanophores, reflecting the key role of *tyrosinase* at the top of the melanin synthesis pathway (Haffter et al. 1996, Kelsh et al. 1996, Page-McCaw et al. 2004). Mice which are mutated at the *Dct* and *Tyrp1* loci have a weaker phenotype with reduced pigment and are dark grey and brown respectively (Bennett et al. 1990). This reflects the importance of these genes in eumelanin synthesis and in maintaining the balance of melanin components (Jackson et al. 1992, Zdarsky, Favor and Jackson 1990). However, whilst candidates have been suggested, we do not currently know the zebrafish phenotype for mutants in these genes and so we do not know the effect on pigmentation in cells which do not express *dct* or *tyrp1b*. Close examination of the correlation between *dct*, *silva*, *tyrosinase* and *tyrp1b* positive cells with each other and with melanin in mutant embryos (by carrying out double and potentially triple ISHs with different combinations of markers) would help to resolve which combinations are conducive to melanin synthesis. Alternatively, for example, it would be interesting to know how numbers of *tyrp1b* positive cells correlate with numbers of melanised cells.

We had also carried out some experiments using xanthophore markers and found that all three of the markers we used, *gch*, *xdh* and *paics*, were expressed in *sox10*<sup>-/-</sup> mutants in a pattern very similar to that seen for the melanophore markers. In wild type embryos there is some overlap between *gch* and melanin and with *xdh* and *mitfa* expression (Parichy et al. 2000b). Assuming these cells do not also express neural markers, this is suggestive of the concept of progressive fate restriction of neural crest cells and thus of partially restricted precursors such as a pigment cell precursor (reviewed in Le Douarin and Dupin (2003)). These partially restricted precursors might be expected to express genes characteristic of all the differentiated cell types. In the process of becoming specified it could be imagined that in wild type embryos cells might exist transiently which express genes characteristic of multiple cell types before markers of all but one cell type are lost and the cell can be described as, for example, a melanoblast. Perhaps when Sox10 function is lost, as in our mutants, we might catch cells in this partially specified state, giving us a window into normal wild type development. To test this idea further double ISHs in wild types and mutants could be used to look for cells with combinations of cell type markers, for example *silva* and *xdh*. Since *silva* and *gch* are so widely expressed in *sox10*<sup>-/-</sup> mutants it would be difficult to imagine that they do not overlap. It is also pertinent to note here some relevant data about iridophores in *sox10*<sup>-/-</sup>



mutants. The expression pattern of an iridophore marker, *ltk*, in *sox10*<sup>3</sup> mutants is also very similar to, and might be expected to overlap with, that of the markers we have used here for melanophores and xanthophores (Lopes et al. 2008). *ltk* expressing cells can be seen in a premigratory position in *sox10*<sup>3</sup> mutants and are increased in number and in strength of expression in mutants as compared to wild types. This differs slightly from the expression of our melanophore differentiation markers, probably because *ltk* appears to have a much earlier role in iridophore development than differentiation, but contributes to the concept of partially restricted precursors caught in this state in *sox10*<sup>3</sup> mutants. Indeed, the authors suggest that these *ltk* expressing cells actually represent multipotent pigment cell precursors.

### 3.3.4 Summary

In this Chapter we have provided evidence in support of our model for the role of Sox10 in melanophores which predicted that melanophore marker genes will be derepressed in *sox10*<sup>3</sup> mutants. We were also able to make a number of other interesting observations from our data about the regulation of our melanophore marker genes and the regulation of melanophore development. We found that xanthophore markers may also be derepressed in *sox10*<sup>3</sup> mutant embryos suggesting the possibility that our model may be generally applicable to other neural crest derived pigment cells. If Sox10 does have a role to inhibit differentiation of melanophores by repressing melanophore differentiation genes then our model predicts that it will have to be downregulated to allow differentiation to proceed. We attempt to test this prediction in Chapter 4.



# Chapter 4

## Testing the Model

### 4.1 Introduction

Our data in Chapter 3 clearly indicated a derepression of multiple melanophore marker genes in *sox10* mutants (Figures 3.03-3.12). This was strongly reminiscent of an observation made in sympathetic neurons in mouse *Sox10* mutants where a neurogenic gene, *Phox2a*, was seen to be derepressed (Kim et al. 2003). The data supports one of the predictions from our model (Figure 1.04), that whilst Sox10 initiates melanophore specification by inducing expression of *mitfa* it also prevents those cells from differentiating by inhibiting expression of genes further downstream of Mitfa. Another of the predictions from our model is that Sox10, due to this repressive function, would need to be downregulated to allow melanophore differentiation to proceed. Loss of *sox10* mRNA from melanophores has been briefly reported by Dutton et al. (2001). They commented that most cells on the lateral pathway at 24 hpf were negative for *sox10* but that at 36 hpf, some melanised cells in the dorsal stripe were still expressing *sox10*. Previous work using a transgenic line has also suggested that there is a downregulation of expression from the Sox10 promoter in differentiating melanophores (Carney 2003). The transgenic line used expressed GFP under the control of a 4.9 kb section of the zebrafish *sox10* promoter, Tg(-4.9*sox10*:GFP). Loss of GFP from differentiating melanophores was observed from approximately 30 hpf. By approximately 90 hpf only a few melanophores still expressed GFP. Of course, due to GFP perdurance, this may not reflect the true timecourse of loss of Sox10 from differentiating melanophores. In our experiments we sought to test the prediction that Sox10 is downregulated in differentiating melanophores more thoroughly. We looked directly at both Sox10 protein, using immunofluorescence (anti-Sox10 antibody kind gift of Dr. B. Appel), and at *sox10* mRNA by ISH, over a timecourse, aiming to provide more comprehensive and conclusive data about the downregulation of Sox10 in melanophores.

In addition to testing our model, if we can establish that Sox10 is downregulated in melanophores and provide details of this phenomenon it will provide a solid foundation for comparisons with mouse melanocyte development. As we discussed in Chapter 1, it has been suggested that mouse and zebrafish may differ significantly in the way that they regulate melanocyte development (Hou, Arnheiter and Pavan 2006). Sox10 appears to be lost from differentiating zebrafish melanophores but it remains to be definitively tested whether Sox10 is downregulated in differentiating melanophores or not. Data that exists from mouse is contradictory, with some reports suggesting that Sox10 is lost from differentiating melanocytes and others that it is not (Chapter 1). Often the work has been carried out in vitro and in melanoma cell lines so the relevance of the work in vivo is also questionable. We hope that by helping fill this gap in our knowledge with respect to zebrafish melanophores we can enable more meaningful comparisons to be made of melanocyte development in both organisms.

Our model predicts that downregulation will be necessary to allow zebrafish melanophores to become fully differentiated. Assuming we could establish that Sox10 is downregulated in melanophores as they differentiate we planned to test this second hypothesis by making a transgenic zebrafish line to drive *sox10* expression in melanophores past the timepoint where it is normally switched off. We would predict that this would either prevent or delay melanophore differentiation. To achieve this overexpression of Sox10 we intended to make two DNA constructs; a control construct containing an appropriate melanophore specific promoter with a reporter gene and an experimental construct with these elements plus the *sox10* gene fused in frame to the reporter gene. These constructs would then be used to generate both transient and germline transgenics which themselves could be used/analysed to determine whether Sox10 downregulation is necessary for melanophore differentiation.

Generating zebrafish transgenics using conventional techniques of simply injecting plasmid DNA into one cell stage embryos, growing these fish and screening them for founders which pass the transgene through their germline, can be very time consuming and laborious and has a low frequency of generating transgenic lines; between about 5 % and 9 % (Stuart et al. 1990, Amsterdam, Lin and Hopkins 1995). Retroviruses have been used to increase the efficiency of gene transfer (Lin et al. 1994) but only increase efficiency to 10 % and have the additional technical difficulties attached to handling retroviruses in the lab (Linney et al. 1999). I-SceI meganuclease has been used to more dramatic effect, increasing efficiency to 30.5 % (Thermes et al. 2002). More recently however, several transposon systems have been developed and used in zebrafish. These include Tc3, Sleeping Beauty and Tol2 (Kawakami 2004, Raz et al. 1998, Davidson et al. 2003). Of these systems it is the Tol2 system (Kawakami 2004) that appears most

promising. The system utilises a medaka transposable element (Tol2) into which the transgene is inserted (Kawakami, Shima and Kawakami 2000). This construct is then co-injected with transposase mRNA (made in vitro) into one cell stage embryos. The transposase RNA is translated into protein in the early embryo. The transposase enzyme cuts the transgene out of the injected construct and allows it to be incorporated into the zebrafish genome. The procedure for generating transgenic lines from this point is the same as for conventional transgenesis except that efficiency is increased many fold (50 % in Kawakami et al. (2004)). For this reason we decided to use this technique for making our transgenics. We were able to obtain, by kind gift of Koichi Kawakami, plasmids containing both the Tol2 element (pT2KXIG, Kawakami et al. (2004)) and the gene encoding a suitable transposase (pCS-TP) and designed our cloning strategy to incorporate our transgene into pT2KXIG. pT2KXIG contained a number of components (Xenopus EF1  $\alpha$  enhancer/ promoter, rabbit  $\beta$ -globin intron, EGFP gene and SV40 polyA signal). Of these, only the SV40 polyA signal was appropriate for our purposes so we planned to remove most of the DNA between the Tol2 elements and replace it with a linker. Linkers are short stretches of DNA which can be designed to contain specific restriction enzyme recognition sites. They are obtained as single stranded DNA, annealed to one another and ligated into a plasmid vector using conventional cloning techniques. The Linker we designed contained all of the restriction sites required for us to be able to insert the different components of our constructs.

There were a number of components to be sourced to generate our constructs. We first needed an appropriate promoter to drive expression in melanophores at all stages of development. An ideal promoter for this purpose would have been the zebrafish *dct* promoter since *dct* is expressed in melanophores from early in their development (19 hpf) to the latest stage observed at 8 dpf (Kelsh et al. 1996). Unfortunately, at the time of commencing the project, the zebrafish genome was not sufficiently well sequenced for us to be able to locate a *dct* promoter region. The *dct* coding region was in fact at the 5' end of a contig with no overlapping stretches of sequenced DNA available that might be expected to contain the *dct* promoter. Instead, we chose to investigate the possibility of using a *Dct* promoter from mouse. We obtained the previously identified mouse *Dct* promoter as a kind gift from Ian Jackson in plasmid pPB2 with a *LacZ* reporter gene (Budd and Jackson 1995, Mackenzie et al. 1997). In mouse, this 3.6 kb promoter had been shown to be active in all areas where *Dct* is normally expressed in the mouse, the early PRE, the telencephalon and in neural crest-derived melanoblasts and melanocytes (Mackenzie et al. 1997). In these transgenic lines, the promoter was also shown to be active in two ectopic sites, the optic nerve and neural crest-derived neuronal cells (DRGs and caudal spinal nerves). We were able to test pPB2 in transient transgenic zebrafish and satisfy ourselves that the mouse

*Dct* promoter was also active in zebrafish and that it recapitulated endogenous *dct* expression. We therefore decided to use this promoter in our constructs.

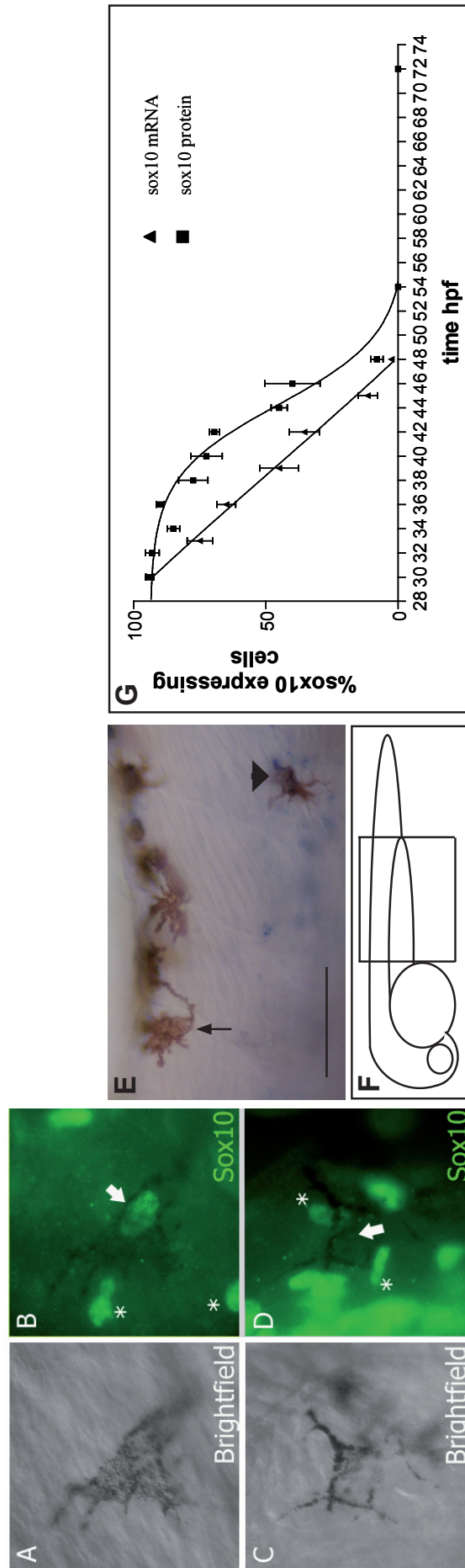
We used the *sox10* coding region from a plasmid called p7.2GFP*sox10* made by James Dutton. To allow us to observe cells expressing ectopic *sox10* and to aid screening of transgenic founders, we proposed to tag our ectopically expressed Sox10 with ECFP by placing *ECFP* (pECFP-C1, Clontech) in frame, upstream of the *sox10* sequence. Previous experiments using a GFP-Sox10 fusion demonstrated that a fusion protein, with the GFP at the N-terminus of the Sox10 protein, was capable of rescuing pigment cells in *sox10* mutants and, therefore, that the Sox10 protein can retain its function even when fused to GFP in this way (J. Dutton, personal communication). We would generate a fusion protein with the ECFP at the N-terminus as opposed to the C-terminus of the Sox10 protein as we were reassured that our CFP-Sox10 fusion was also likely to be functional. A second line expressing only ECFP under the control of the *Dct* promoter would be useful as a control for our experiment but could also become a useful tool in its own right. By choosing to use ECFP, this line could be crossed with available lines expressing GFP in the neural crest for use in any number of experiments where labelling neural crest and early melanoblasts in distinct colours would be required.

## 4.2 Results

### 4.2.1 Sox10 is Downregulated in Differentiating Melanophores

We performed *sox10* in situ hybridisation and Sox10 antibody staining (antibody kind gift from Dr. B. Appel) on embryos that had not been treated with PTU so that melanophores were identifiable by their pigment. For the ISHs, we used embryos at 30 hpf and at three hour intervals after that until 48 hpf. For the antibody staining we used embryos at 30 hpf and at two hour intervals until 48 hpf, plus 54 hpf and 72 hpf. In both experiments, the embryos were scored blind so that we did not know the exact age of the embryos at the time of scoring. At each stage, 20 pigmented melanophores in the region of the trunk between the edge of the yolk and the end of the yolk sac extension (Figure 4.01) were scored in each of five embryos for whether or not they were expressing Sox10/*sox10*. We attempted to score melanophores from different dorso-ventral and antero-posterior positions. Both experiments were repeated twice; visual inspection of the data showed that each set was consistent with the other and so data were pooled.

We showed that the loss of Sox10 protein from pigmented melanophores occurs between 32 hpf and 54 hpf (Figure 4.01). Loss of Sox10 from melanophores was initially slow



**Figure 4.01 Sox10 and *sox10* are downregulated in differentiating melanophores**

A-D. Sox10 antibody staining at 33 hpf. A, B. Pigmented melanophore seen in brightfield illumination (A) showed Sox10 immunofluorescence (B, arrow). C, D. A pigmented melanophore in the same embryo seen in brightfield illumination (C) which was negative for Sox10 (D, arrow). Asterisks indicate other Sox10 positive cells which may be melanoblasts or a different cell type all together.

E. Brightfield image of a dorsal section of the trunk of a 33 hpf embryo which was processed by in situ hybridisation for *sox10*. Some melanophores are negative for *sox10*, arrow, where as others (e.g. arrowhead) are clearly positive for *sox10*. Scale bar 50  $\mu$ m.

F. Area of embryo in which melanophores were scored.

G. Timecourse of Sox10 and *sox10* in posterior trunk melanophores, mean  $\pm$  SE of counts from two experiments.

until 36 hpf, when it accelerates. The time at which half of the melanophores were Sox10 positive and half were negative was at approximately 44 hpf. In contrast, rate of loss of *sox10* transcript from melanophores was constant and more rapid. The time at which half of the melanophores scored were *sox10* positive and half were negative was 38 hpf, six hours earlier than for Sox10 protein. We have shown that Sox10 and *sox10* are downregulated in melanophores as they differentiate, both confirming and extending previous work.

### **4.2.2 Is Sox10 Downregulation Necessary for Melanophore Differentiation?**

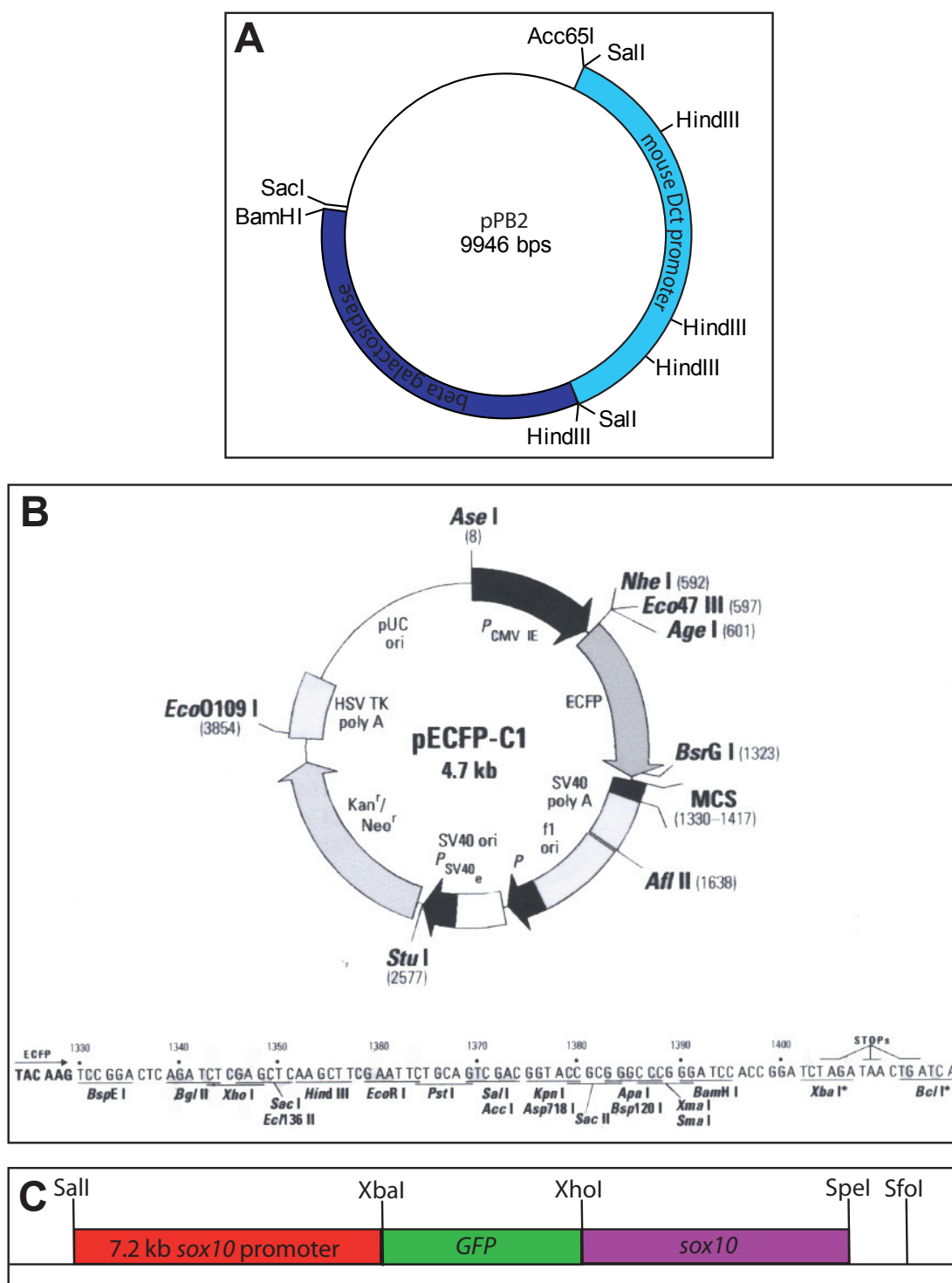
We now wanted to test whether downregulation of Sox10 is necessary for melanophores to differentiate by generating a transgenic line which expresses Sox10 in melanophores past the timepoint where it would normally be downregulated. We aimed to generate two constructs in a Tol2 vector backbone; an experimental plasmid containing the 3.6 kb mouse *Dct* promoter with an *ECFP-sox10* fusion downstream of the promoter, and a control plasmid with only *ECFP* downstream of the promoter. These plasmids could then be used to generate stable transgenic lines which could be used to address our hypothesis. Information on all plasmids used can be found in Figures 4.02 and 4.03.

#### **4.2.2i Mouse *Dct* Promoter Activity in Zebrafish Embryos**

To determine whether the mouse *Dct* promoter might be suitable for our proposed use in zebrafish we had to test whether the promoter was active in zebrafish and if so, whether it gave expression in melanophores. The plasmid pPB2 contains 3.6 kb of the mouse *Dct* promoter with a *LacZ* reporter gene downstream of it. We injected 9.2 nl of this plasmid at a concentration of 40 ng/μl into zebrafish eggs at the one cell stage, grew them to approximately 27 hpf, fixed them lightly and detected β-galactosidase activity using XGal. For the perdurance of the β-galactosidase, any cells in which the mouse *Dct* promoter had been active would then be labelled with a blue precipitate. These cells could be observed and scored by position and morphology to determine the cell types in which the promoter had been active.

We obtained compatible results from three separate sets of injections and so data have been combined here. Approximately 1420 eggs were injected over the three experiments, although only 648 (46 %) of the injected embryos survived to 27 hpf. This high death rate was expected due to the high concentration of DNA which we had used to maximise the concentration of DNA in individual cells and integration of the DNA into the genome to maximise our chances of observing LacZ positive cells in surviving embryos (Stuart, McMurray and Westerfield 1988). 18 % of fish



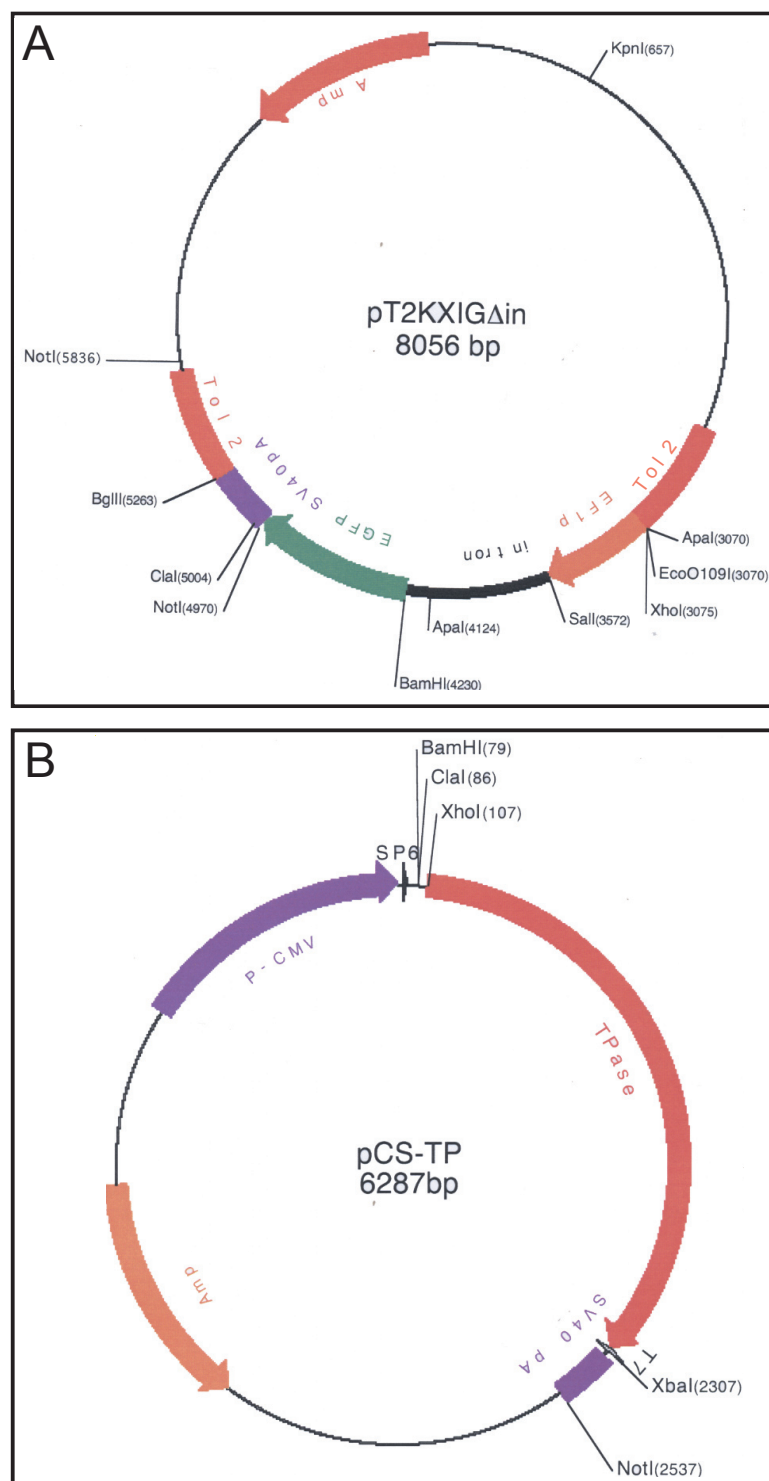


**Figure 4.02** Plasmids from which components were taken for creation of pTol2DctCFPsox10

A. pPB2 from which the mouse *Dct* promoter was removed as a SalI fragment.

B. pECFP-C1 from which *ECFP* was removed as an AgeI/BspEI fragment (diagram taken from plasmid data sheet, Clontech).

C. p7.2GFPsox10 was made with a pCS2 backbone. We were able to remove the *sox10* coding region as an XhoI/SfoI fragment using the XhoI site present at the 5' end of the *sox10* (created during the original cloning) and the SfoI(KasI) site in the multiple cloning site of CS2.



**Figure 4.03 Tol2 system plasmids**

A. pT2KXIG contains the Tol2 elements and is the vector from which pTol2DctCFP and pTol2DctCFPsox10 were built. Sequence between the Tol2 elements and sites ApaI (3070) and ClaI (5004) was removed.

B. pCS-TP contains the gene for the transposase which is used in conjunction with the pT2KXIG plasmid to aid integration of our construct into the genome. pCS-TP is cut with NotI and used with SP6 RNA polymerase to make transposase RNA which is co-injected with our constructs into one cell stage eggs.

Plasmid diagrams adapted from those sent by Koichi Kawakami.

that survived to 27 hpf had LacZ positive cells. Fish had between one and 16 cells positive for LacZ. The location and cell type of each of 144 LacZ positive cells from 54 embryos at 27 hpf were noted (Table 4.01). Most of the cells observed in this way (57.8 %) could either be positively identified as melanophores (43.2 %, either neural crest derived or in the pigmented retinal epithelium) or, by morphology and location, could be identified as neural crest derived cells (14.6 %, Table 4.01, Figure 4.04). 13.2 % of the cells observed were muscle cells, clearly identifiable by their characteristic shape and exact size of one somite in length. Many transgenes generate ectopic expression and often, as in this case, this ectopic expression is in the muscle of transient transgenics (Thummel, Burket and Hyde 2006, Carney 2003, Udvardi and Linney 2003) so this observation did not concern us. 4.9 % of cells were in the CNS. These cells were in different areas of the CNS, from the forebrain/telencephalon to the spinal cord. *Dct* is expressed in the mouse telencephalon but not at all in the zebrafish brain and it would be reasonable to assume that there are differences between the control elements present in the respective promoters which orchestrate this. Perhaps these differences mean that when in zebrafish, the mouse *Dct* promoter can drive expression of a reporter in the telencephalon or even other areas of the CNS. Alternatively, the expression may be a result of the transgene location; maybe close to a CNS enhancer. True ectopic expression was seen in 4.6 % of cells in the otic epithelium or the olfactory epithelium. However, this was a small percentage of cells and was not deemed likely to cause a problem in our experiments. The remaining 20.1 % of cells were either unidentifiable or were apoptotic (apoptotic cells were identified by their characteristic ‘blebbed’ appearance).

From these experiments we concluded that the mouse *Dct* promoter had the correct spatiotemporal properties and would be a suitable promoter for us to use in our construct.

#### **4.2.2ii Preparing the Backbone**

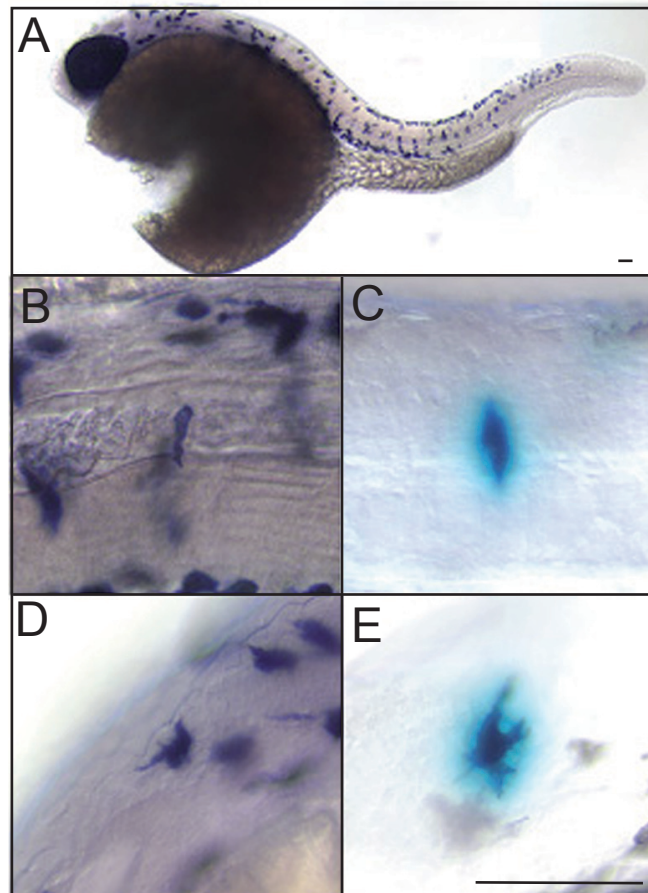
The scheme we designed for the building of our two constructs is detailed in Figure 4.05. First, approximately 5 µg pT2KXIG was cut using *ApaI* and *ClaI* enzymes in a Multicore buffer (Promega). The whole digest was run on a 1 % agarose gel and the 6 kb band, corresponding to the ‘empty’ pT2KXIG vector, was excised from the gel and gel purified (Figure 4.05 A).

In order to be able to build our construct into this empty pT2KXIG vector we designed a linker. We designed our linkers to contain *Sall*, *AgeI*, *BspEI*, *XhoI* and *KasI* restriction sites which we would use later to construct our full construct. To aid ligation into the empty pT2KXIG vector the linkers were also designed to have *ApaI* and *ClaI* sticky

Table 4.01 LacZ expression in transient transgenics

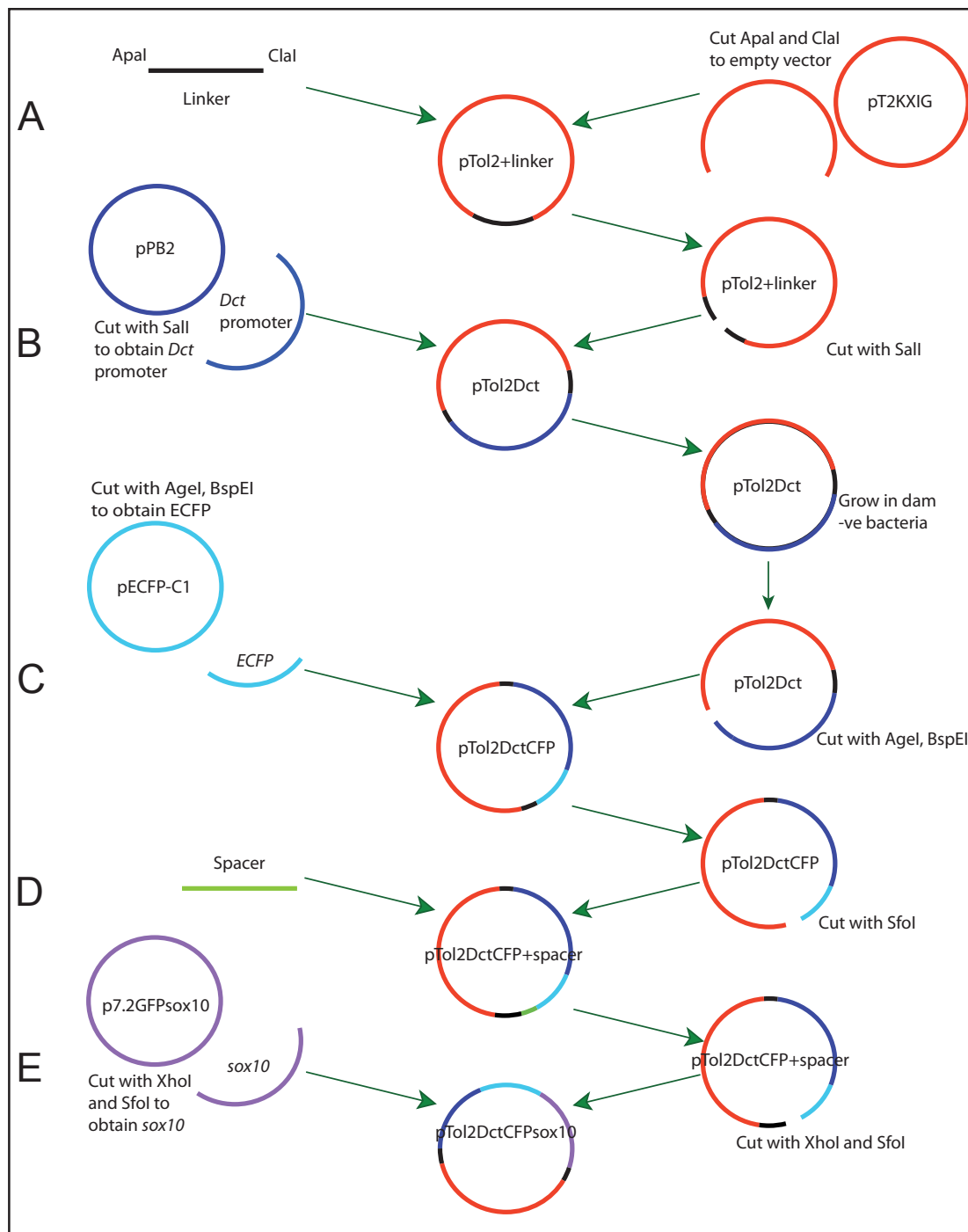
Cell Type	No. LacZ positive cells	% LacZ positive cells
<b>Expected expression</b>		
Pigmented melanocyte	9	6.3
Probable melanocyte	42	29.2
Neural crest-like	21	14.6
Pigmented Retinal Epithelium	8	5.6
Choroid	3	2.1
<b>Subtotal expected</b>	<b>83</b>	<b>57.8</b>
<b>Ectopic expression</b>		
Ear	3	2.1
Olfactory epithelium	3	2.1
CNS	7	4.9
Muscle	19	13.2
Apoptotic	17	11.8
Unknown	12	8.3
<b>Subtotal ectopic</b>	<b>61</b>	<b>42.4</b>
<b>Total</b>	<b>144</b>	<b>100</b>

The plasmid pPB2 was injected into one cell zebrafish embryos. Fish were evaluated for LacZ expression at 27hpf. Most of the LacZ expression was specific to cells which endogenously express *dct*. Note: percentages do not total 100 due to rounding.



**Figure 4.04 Mouse *Dct* promoter recapitulates endogenous *dct* expression**

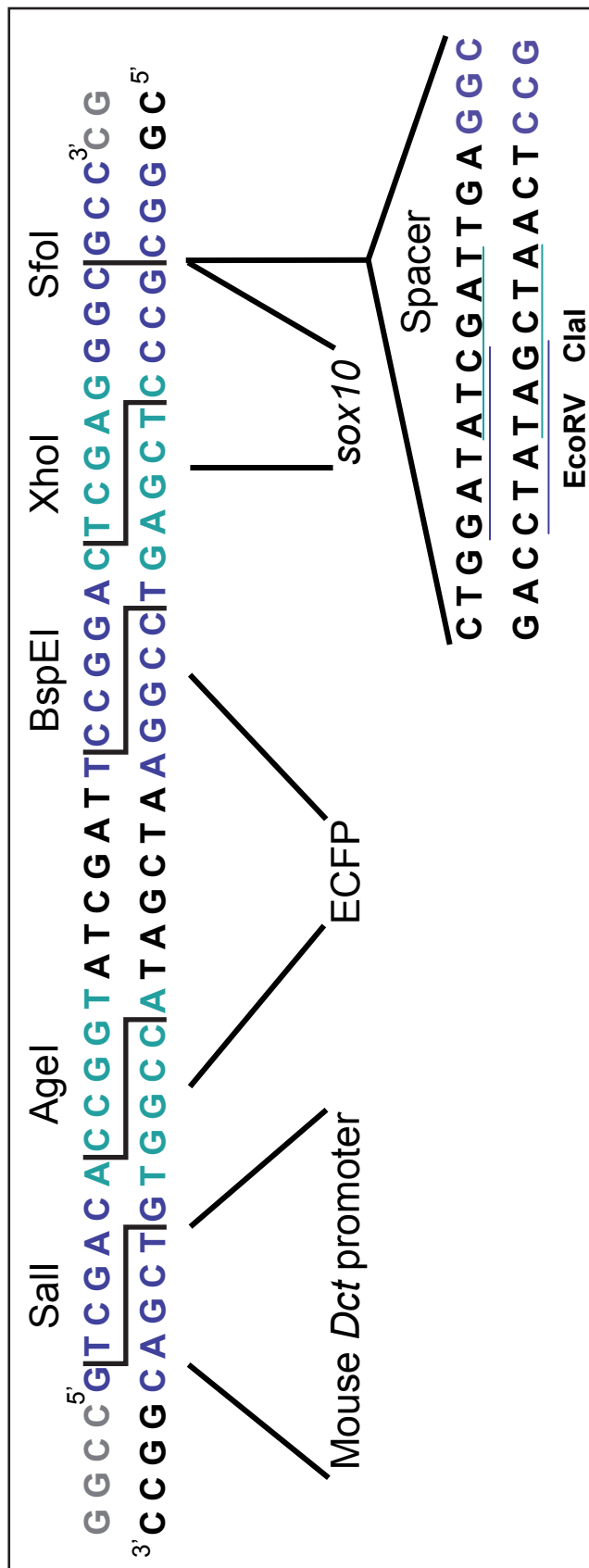
The plasmid pPB2 was injected into one cell zebrafish embryos. Fish were evaluated for LacZ expression at 27hpf. Panels A-E compare the wild type *dct* in situ hybridisation pattern (A, B and D) with examples of LacZ expressing cells in fish injected with pPB2 (C and E) Scale bar is the same for A and for B - E, 50µm.



**Figure 4.05 Schematic of cloning strategy**

Each component has and retains its own colour throughout. A. Preparing the vector. B. Inserting mouse *Dct* promoter. C. Inserting *CFP* sequence. D. Inserting a spacer sequence. E. Inserting *sox10* sequence. For more details see text.





**Figure 4.06 Representation of the linker inserted into pT2KXIG into which our construct was built**

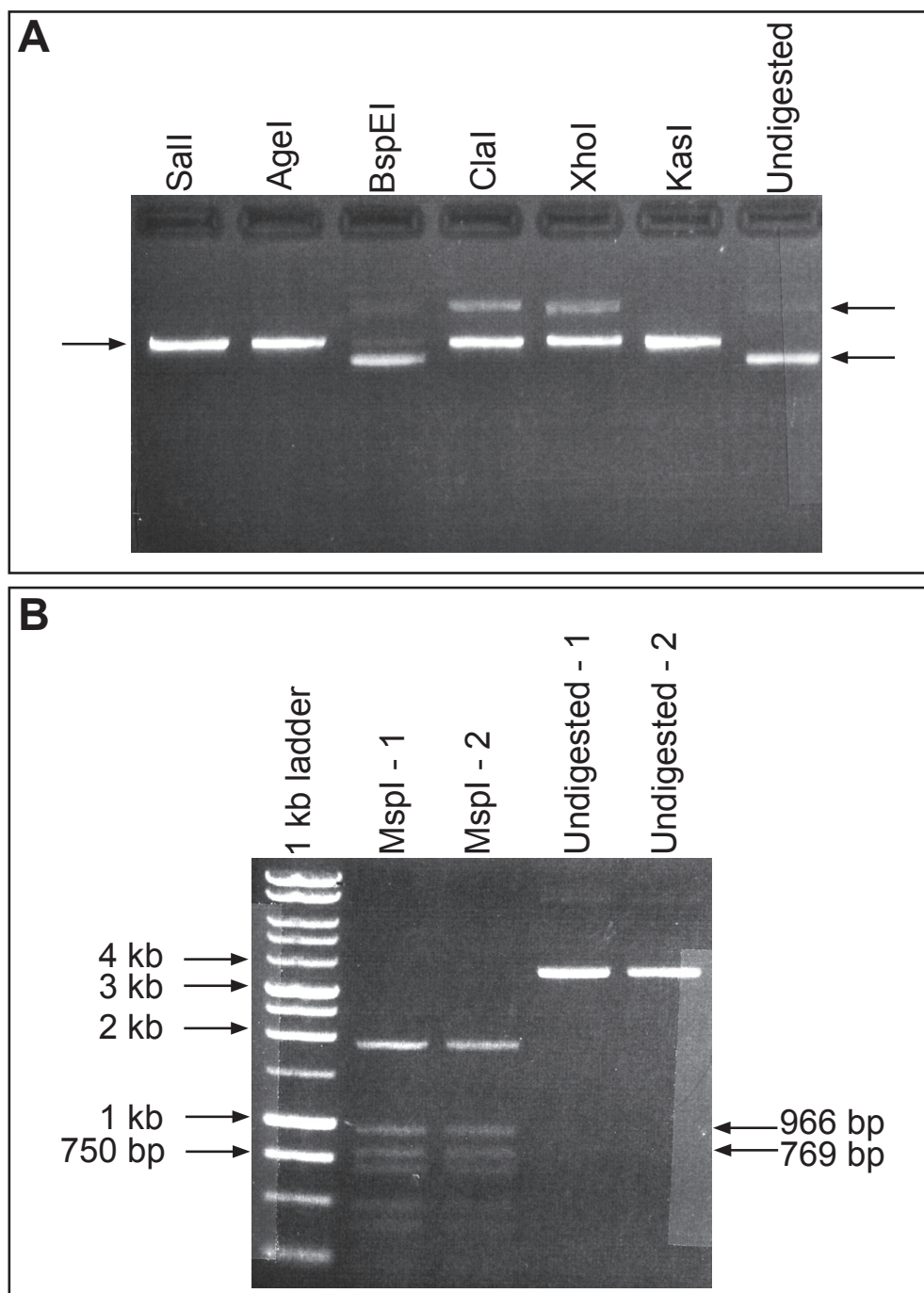
Shown are the restriction sites used (highlighted in blue) and the ends of pT2KXIG into which the linker was ligated (grey). NB. The Apal and ClaI restriction sites used to create these overhangs in the pT2KXIG are not recreated in the pTol2+linker plasmid. Also indicated are the different fragments that were inserted into the Linker and the restriction enzyme sites which were used for this. A Spacer was inserted into the SfoI site to separate it from the XhoI site and allow the insertion of the *sox10* gene. Note that KasI and SfoI have the same recognition site but only SfoI is shown here. The Spacer sequence is indicated as well as the EcoRV and ClaI sites used to test for the presence of the Spacer (underlined in blue). Note also that the SfoI recognition site is only recreated at one end of the Spacer (purple).

ends when annealed to one another (Figure 4.06). Linkers 1 and 2 were ordered from Invitrogen as a 200 nmol synthesis of custom primers and were PAGE purified prior to being sent to us (see Appendix for sequences).

Linkers 1 and 2 were annealed to each other (Chapter 2). A ligation was then set up using 3 µl annealed Linkers and 1 µl empty pT2KXIG vector. The ligation was used to transform competent cells, two of the resulting colonies were picked and minipreped. Digests were set up to check for the presence of Sall, AgeI, BspEI, XhoI and KasI restriction sites. All except BspEI were present (Figure 4.07). We hypothesized that the BspEI digest failed because the activity of BspEI is blocked by overlapping *dam* methylation. Since the cells into which the plasmid had been transformed were *dam* positive, if we had inadvertently created a methylation site in our linker it would be methylated and BspEI would be unable to cut the DNA despite the presence of a BspEI restriction site. MspI is an enzyme which recognises the same restriction site as BspEI but is not methylation sensitive. We therefore digested our plasmid with MspI to test for the presence of the BspEI site. MspI was able to cut the plasmid in several places. If our BspEI site is present in the linker among other band sizes there will be bands of 769 bp and 966 bp. If the site is not present, these bands will not be present but a 3696 bp band will be. The band pattern we saw after MspI digestion included bands of the expected sizes as well as bands of 769 bp and 966 bp but not of 3696 bp (Figure 4.07). From this, we were able to determine that MspI was cutting within our linker and that the BspEI site was therefore present in our linker. We were now confident that our complete linker was present within the pT2KXIG backbone and the plasmid was named pTol2+linker. To confirm that methylation was preventing BspEI cutting our plasmid, and to find a way to remove that methylation so that we could use the BspEI site for our cloning, we transformed pTol2+linker into SCS100 cells (Stratagene). These cells are *dam* negative and thus will generate unmethylated DNA. It was possible to cut pTol2+linker purified from these cells with BspEI, confirming that the BspEI site was present and that it had not cut before due to methylation. This showed that we would be able to use the BspEI site in later cloning steps if the plasmid had been prepared from *dam* negative cells.

#### **4.2.2iii Inserting the Mouse *Dct* Promoter**

The mouse *Dct* promoter fragment was isolated from pPB2 as a Sall fragment by digesting approximately 5 µg pPB2 with Sall, running the whole digest on a 1 % agarose gel, removing the 3.6 kb band corresponding to the mouse *Dct* promoter and gel purifying the DNA (Figure 4.05 B). 5 µg pTol2+linker (methylated) was linearised using Sall, run on a 1% agarose gel and gel purified. A ligation was set up using 2 µl linearised pTol2+linker and 3.5 µl mouse *Dct* promoter with Sall ends and XL-1 Blue



**Figure 4.07 Successful insertion of Linker into pT2KXIG**

A. Digests were run against undigested plasmid as a marker. pTol2+linker can be digested with Sall, AgeI, ClaI, XhoI and KasI. The plasmid appears to linearise producing single bands for most of these enzymes (left arrow). DNA digested with ClaI and XhoI appear to be incompletely digested, but a band equivalent in size to that produced from the other digests is present as well as one larger, undigested band (top right arrow). The DNA appears undigested with BspEI.

B. BspEI is methylation sensitive. MspI cuts at the same recognition site but is not methylation sensitive. If the BspEI site is not present, cutting with MspI will give a band at approximately 3.7 kb; if it is, bands will be seen at 769 bp and 966 bp instead. No band is present at 3.7 kb but bands are present at 769 bp and 966 bp. Therefore the BspEI site is present in our linker.

cells (*dam* positive, Stratagene). 17 colonies were miniprepmed and digested with *Sall* to ascertain whether the promoter had successfully inserted. The expectation was to see two bands, one being the 6 kb pTol2+linker and one the 3.6 kb mouse *Dct* promoter. However, only the 6 kb pTol2+linker band was present and we concluded that under the conditions used the plasmid had simply re-annealed to itself without incorporating the mouse *Dct* promoter. To prevent this, we treated the linearised pTol2+linker with Shrimp Alkaline Phosphatase (SAP) to remove the phosphates from the 5' ends of the DNA. This would prevent the ends from annealing to one another but still allow them to anneal to untreated DNA ends. In 40  $\mu$ l of plasmid, we had 0.3 pmol of ends and needed at least 0.03 units SAP (0.2 units were actually used as less was too little to measure accurately). Following SAP treatment a second ligation was set up, this time using 1  $\mu$ l linearised and SAP treated pTol2+linker and 3  $\mu$ l mouse *Dct* promoter with *Sall* ends. However, even after a number of attempts no colonies were produced using this method. The reasons for this are unclear and it could have been a result of a combination of a number of factors including the difficulty of inserting a comparatively large fragment into the vector (3.6 kb into 6 kb).

We reverted to using colonies generated from ligations between linearised pTol2+linker that had not been treated with SAP and the mouse *Dct* promoter. To search these colonies in larger numbers than could otherwise be done for the few that might contain the insert we used Colony PCR. We prepared DNA from colonies and carried out a PCR using primers BS1 and TYR1 (Kawakami 2004) which bind to the Tol2 elements within the plasmid, one either side of our insert. We predicted that we would see a product if the insert was not present but would not if it was since the interval between the primers would then be too large to amplify. This method would generate a small number of false positives if DNA preparation was not efficient and PCR failed on these samples (we could control for poor PCR results by using undigested pTol2+linker as a positive control) but it would at least reduce the number of colonies which would need to be miniprepmed and digested to identify the required plasmid. After screening 144 colonies in this way and miniprepping 30 of these, we identified one colony which, when digested with *Sall* gave three bands corresponding to linear plasmid plus insert (~10 kb, present as a result of incomplete digestion of the plasmid), pTol2+linker backbone (6 kb) and mouse *Dct* promoter (3.6 kb). We performed a second digest to determine the orientation of the promoter within the vector as the cloning was non-directional and only one orientation would be useful. When digested with *KpnI*, if the insert was in the correct orientation we would see bands of 4.7 kb and 5 kb, in the incorrect orientation the digested plasmid would produce bands of 6.2 kb and 3.4 kb, which is unfortunately what we saw. This meant that the promoter was inserted in the incorrect orientation but we now knew that the promoter definitely could insert into

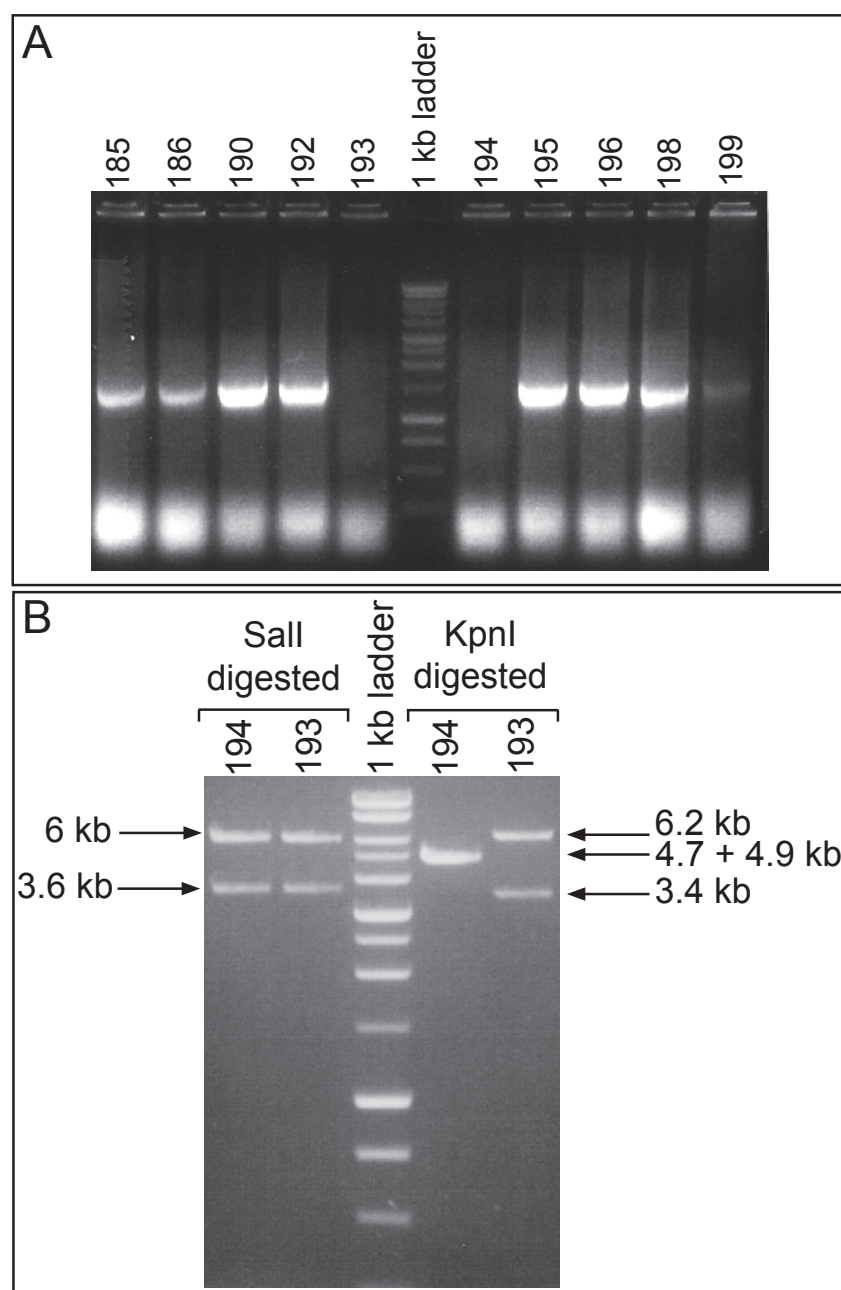
the vector (presumably its large size made the event less likely). So we continued with our Colony PCR strategy. After doing Colony PCR on a further 201 colonies, 12 more minipreps yielded a further two colonies in which the promoter was inserted in the incorrect orientation, but it also yielded one colony in which the promoter was in the correct orientation (colony number 194, Figure 4.08). This plasmid was now named pTol2Dct.

#### **4.2.2iv Inserting the *ECFP* Gene**

*ECFP* was excised from approximately 5 µg of the pECFP plasmid using AgeI and BspEI enzymes (Figure 4.05 C). These two enzymes do not work in the same buffer so the plasmid was digested with AgeI first and cleaned by Phenol Chloroform extraction before being digested with BspEI. The digest was run on a 1 % agarose gel and the desired 800 bp band containing *ECFP* was removed from the gel and the DNA extracted from it. pTol2Dct was transformed into SCS110 *dam* negative bacteria to produce unmethylated DNA so that the plasmid could then be linearised with AgeI and BspEI. Since the sequence of pECFP close to the BspEI recognition site (methylation sensitive) was such that it would not be methylated, the BspEI enzyme was able to cut pECFP without the need to ensure the DNA was unmethylated. 5 µg unmethylated pTol2Dct was first digested with BspEI. After cleaning the DNA with a Phenol Chloroform extraction it was digested with AgeI. The DNA was again cleaned using a Phenol Chloroform extraction (the small fragment of linker that was removed by AgeI/BspEI digestion was lost during Phenol Chloroform extraction).

Both BspEI and AgeI give CCGG overhangs so it was more likely that the pTol2Dct backbone would re-ligate to itself rather than incorporate the *ECFP* fragment. To prevent this approximately 0.5 µg linearised pTol2Dct was SAP treated using 0.2 units of SAP and a ligation was set up using 1 µl of this DNA and 10 µl *ECFP* fragment. This ligation was microdialysed and the whole quantity used to transform competent cells. 18 colonies were picked and miniprepped. Of course, since AgeI and BspEI ends are compatible with each other, the *ECFP* fragment may be inserted in either direction, only one of which was the one we wanted. To determine which samples contained the *ECFP* insert in the correct orientation each was digested with AgeI. Plasmids containing the *ECFP* fragment in the correct orientation would be linearised by AgeI because in this case the restriction site is remade. If however, the plasmid has annealed to itself and does not contain the *ECFP* fragment or if the *ECFP* fragment has inserted in the incorrect orientation, it will not linearise, since when AgeI and BspEI ends ligate to one another they do not remake the AgeI restriction site. Of the 18 colonies picked, 14 could be digested by AgeI and appeared to be larger than 10 kb (we expect a 10.4 kb plasmid at this stage) confirming that these colonies contain the



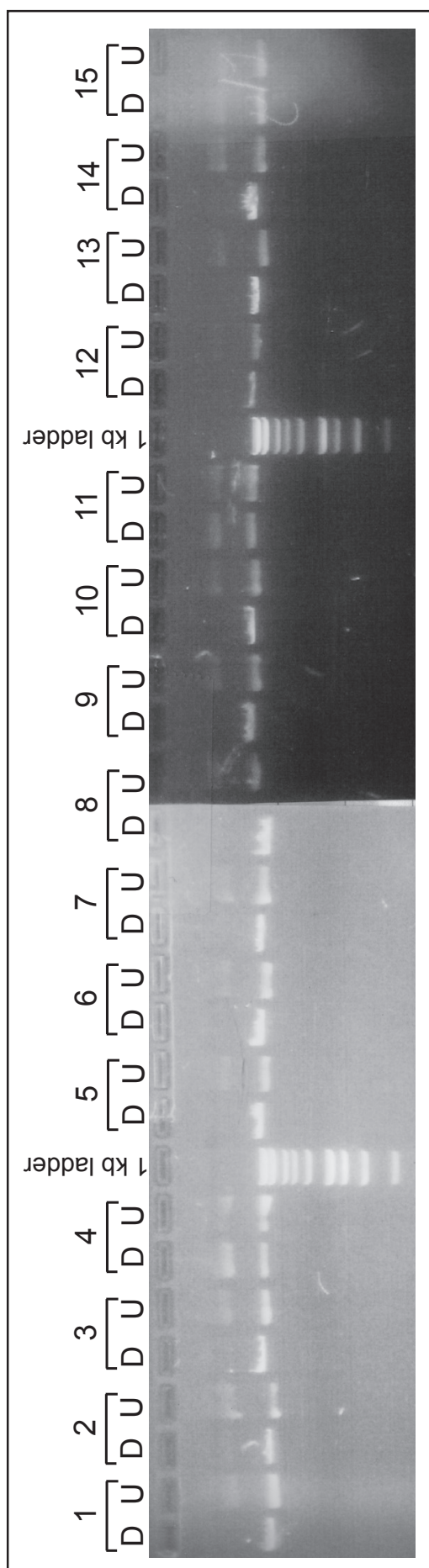


**Figure 4.08 Cloning of the mouse *Dct* promoter into pTol2+linker**

A. Colony PCRs using BS1 and TYR1 primers on colonies 185, 186, 190-196, 198 and 199. All colonies except 193 and 194 give products using this PCR. This indicates that the colonies from which these samples came do not contain the mouse *Dct* promoter as this product would be too large to be made. That 193 and 194 do not produce a PCR product indicates that they may have incorporated the mouse *Dct* promoter.

B. Digests to confirm that the mouse *Dct* promoter has inserted into the plasmids and to determine the orientation of the insert. *SalI* digests confirm the presence of the promoter. In both colonies 193 and 194 the enzyme digests out the promoter giving a 6 kb pTol2+linker band and a 3.6 kb promoter band. Digests with *KpnI* determine the orientation of the promoter in the plasmid. 193 gives two bands of 6.2 kb and 3.4 kb, the expected sizes if the insert is in the incorrect orientation. 194 gives one band at approximately 4.7-4.9 kb, the size expected if the promoter is inserted in the correct orientation. Note that this band is brighter than the others since it consists of two fragments of DNA.





**Figure 4.09 Cloning of *ECFP* into pTol2Dct**

Minipreps from 18 different colonies were digested with AgeI, 15 are shown here. U=undigested, D=digested. If the plasmid was not linearised with AgeI it indicated that the plasmid did not contain the *ECFP* insert or did not contain the *ECFP* insert in the correct orientation. Only samples 4, 11 and 15 did not digest with AgeI, note the presence of an upper band even in the digested sample and that the lower band in the digested sample is the same size as for the undigested sample. The single band present in linearised samples (e.g. 13) is larger than the lower band of the undigested sample. Samples 1-3, 5-10 and 12-14 have only one band in the digested lane representing linearised plasmid, therefore, these contain *ECFP* in the correct orientation.

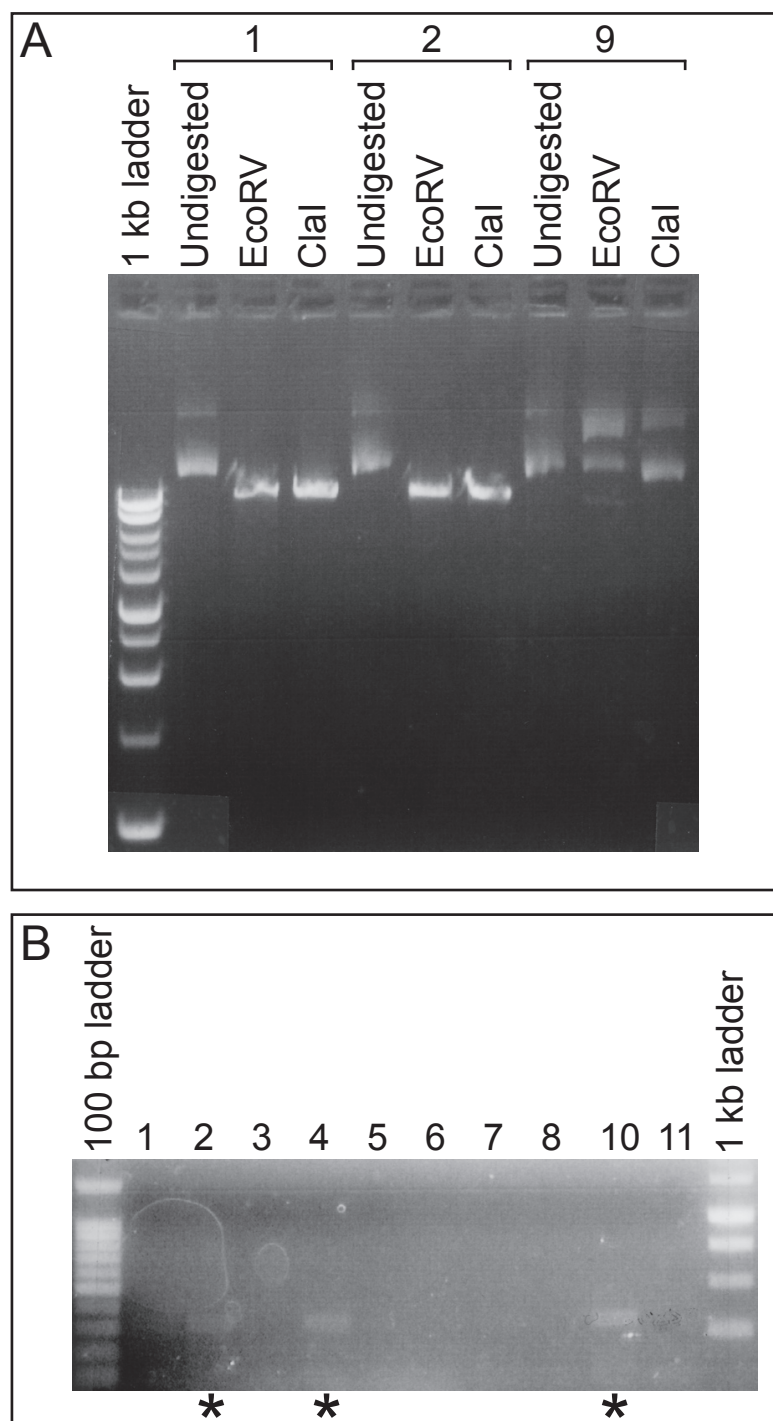
*ECFP* insert in the pTol2Dct plasmid (Figure 4.09). Sample 2 was chosen and named pTolDctCFP.

#### **4.2.2v Inserting the *sox10* Gene**

The *sox10* gene was isolated from the p7.2GFP*sox10* plasmid using *KasI* and *XhoI* in separate digests (since their buffers were incompatible). p7.2GFP*sox10* was first cut with *KasI*, cleaned and then digested with *XhoI* (Figure 4.05 D). The digest was run on a 1 % agarose gel, the appropriate band excised and the DNA cleaned by gel extraction. 5 µg pTol2DctCFP was linearised first with *KasI*, cleaned by Phenol Chloroform extraction and then cut with *XhoI* before being cleaned again by Phenol Chloroform extraction. 113 colonies were tested using Colony PCR and S21/S22 primers (see Appendix) which bind to the *sox10* gene. However, none gave products and it was concluded that no colonies had the *sox10* gene in the pTol2DctCFP plasmid.

When pTol2DctCFP has been cut with *KasI* there is only 1 bp between the *XhoI* site and the end of the DNA strand. Whilst this should not greatly affect the ability of the enzyme to cut the DNA (Moreira and Noren 1995) it is possible that a low frequency of *XhoI* cutting the pTol2DctCFP plasmid would explain why we did not find any plasmids containing our *sox10* insert. If *XhoI* is not cutting efficiently, then any colonies we obtain will just contain pTol2DctCFP which has been cut with *KasI* and has re-ligated to itself. To solve this problem we decided to insert a spacer into the plasmid between the *KasI* site and the *XhoI* site. We designed two oligos such that, if pTol2DctCFP is cut with *SfoI* (*SfoI* has the same recognition site as *KasI* but cuts to give blunt ends) and the annealed oligos, now called the Spacer, inserted into it then the *KasI/SfoI* site is remade only at the end furthest away from the *XhoI* site (see Appendix). The Spacer also contained unique *ClaI* and *EcoRV* sites to enable us to check for its presence. A primer (spacerprimer, see Appendix) was designed over the Spacer and adjacent sequence which, in conjunction with the BS1 primer, would also allow us to check the orientation of the Spacer. The oligos to make the Spacer were ordered as for the Linker.

Thus, approximately 5 µg pTol2DctCFP was cut with *SfoI* and cleaned by Phenol Choloform extraction. The spacer oligos were diluted to 220 µM and annealed to one another as described in Chapter 2 for the Linkers. Then, 7 µl of Spacer and 1 µl *SfoI* linearised pTol2DctCFP were used in a ligation. 11 of the resulting colonies were picked, 10 of which could be digested with *EcoRV* and *ClaI* and so contained the Spacer. Three of these colonies also produced products using the Spacer and BS1 primers in a PCR and so must contain the Spacer in the correct orientation (Figure 4.10). One of these was chosen and the plasmid named pTol2DctCFP+spacer.

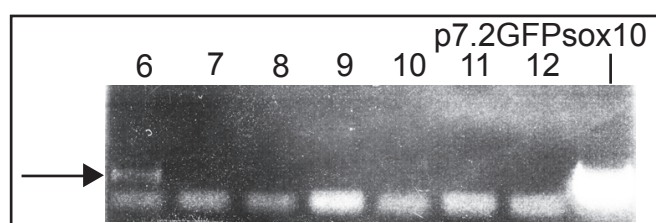


**Figure 4.10 Correct insertion of spacer**

A. EcoRV and ClaI will only cut in the spacer, thus linearising the plasmid. To determine whether any contained the spacer, 11 colonies were miniprepmed and digested with these enzymes. Samples 1, 2 and 9 are shown here as examples. All samples except sample 9 were linearised with both EcoRV and ClaI showing that all except 9 contained the spacer.

B. To determine the orientation of the spacer a PCR was carried out on diluted miniprepmed DNA from samples 1-8, 10 and 11 using spacerprimer and BS1. Samples 2, 4 and 10 gave products (\*) indicating that they have the spacer inserted in the correct orientation for the subsequent cloning step.

pTol2DctCFP+spacer was linearised with *KasI*, Phenol Chloroform extracted and cut again with *XhoI* (Figure 4.05 E). The cut plasmid was cleaned by Phenol Chloroform extraction and a ligation set up with 2 µl pTol2DctCFP+spacer and 6 µl *sox10* insert. Of 57 colonies analysed by PCR using S21/S22 primers for the *sox10* insert (see Appendix for sequences) none gave a band of the correct size but four generated a product of the incorrect size. *MluI* and *NdeI* would both be expected to linearise the plasmid if the *sox10* gene is present correctly in the construct. DNA from the four colonies which gave incorrect products from the PCR with S21/S22 primers did not cut as expected with *MluI* and *NdeI*. Two samples were cut by neither enzyme and the other two cut with only one enzyme. So we suspected that there may be contamination of our *sox10* fragment with other fragments of DNA which appeared to be incorporating into our construct instead of the *sox10* DNA. Further, careful analysis of p7.2GFP*sox10* digested with *KasI* in larger quantities than had been examined before revealed faint bands of unexpected sizes which had not been noticed before (data not shown). This confirmed our idea that contamination of the *sox10* insert had occurred. These bands were not seen when the plasmid was digested with *SfoI*. It is likely that this is because *SfoI* is methylation sensitive and the sites where *KasI* cuts unexpectedly to give unexpected bands are methylated so that *SfoI* does not cut there but only at the site at the 3' end of the *sox10* gene. We decided to isolate more *sox10* DNA using *SfoI* and *XhoI* instead of *KasI* and *XhoI*. A small quantity of the insert was used in a PCR with S21/S22 primers to confirm that *sox10* was definitely present. A ligation was set up using this DNA and pTol2DctCFP+spacer that had also been cut with *SfoI* and *XhoI*. Of 12 colonies picked and minipreped, one gave a band of expected size when used in a PCR with S21/S22 primers for the *sox10* gene (Figure 4.11). Subsequent restriction digest analysis was performed to confirm that this plasmid contained the *sox10* insert (data not shown). Digestion with *XhoI* and *SfoI* removed the *sox10* fragment again. *NheI* linearised the plasmid, as expected since *sox10* contains an *NheI* recognition site but no other components of the construct did. *Sall* and *AgeI* digests also produced bands of expected sizes consistent with the idea that we had incorporated the *sox10* gene into our construct. Primers BS1, S22, S19 and seqCFP were used as sequencing primers to verify the whole sequence between the beginning of the *CFP* sequence and the end of the *sox10* sequence (Figure 4.12). We named the plasmid pTol2DctCFP*sox10* (Figure 4.13). The same sequencing data enabled us to confirm that the junctions of the *sox10* sequence with the other cloned components were correct. Particularly important was that the *sox10* sequence was in frame with the *CFP* sequence to produce a single fused protein and we found that this had occurred as expected (Figure 4.12).



**Figure 4.11 Correct insertion of *sox10* into pTol2DctCFP to create pTol2DctCFPsox10**

DNA from colony number 6 generates a band of expected size (compare to p7.2GFPsox10 plasmid marker/control) when used in a PCR with primers S21 and S22 suggesting *sox10* has inserted to the plasmid in this colony. Later data using restriction enzyme digestion supports this (data not shown).

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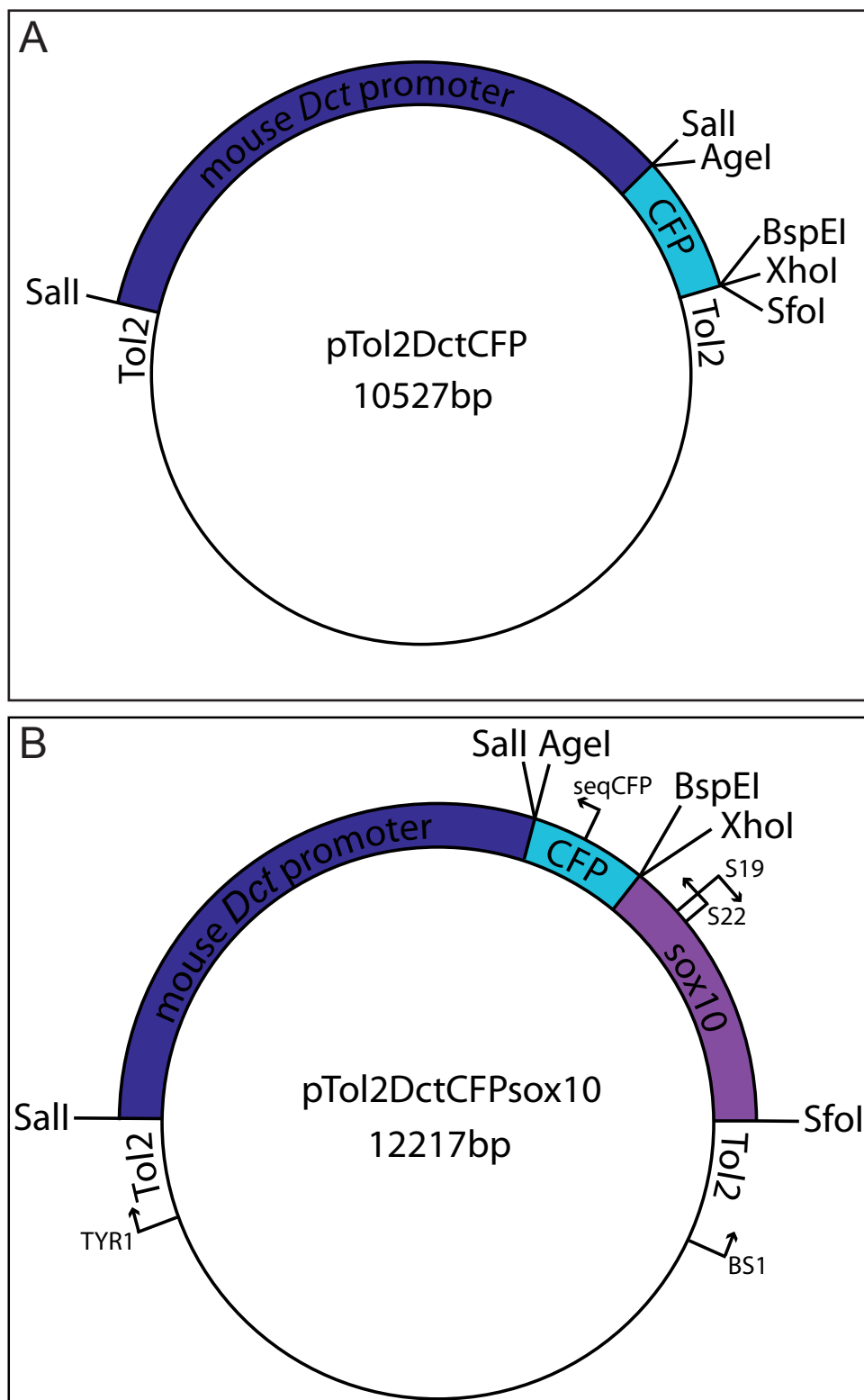
      M V S K G E E L F T G V V P I L V E L D G D V N
ccggtcgccaccatgtgtgagcaagggcgaggagctgttcaccgggtggtgccatcctggtcgagctggacgcgacgtaaac
G H K F S V S G E G E G D A T Y G K L T L K F I C T T G
ggccacaagtgcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgacctgaagttcatctgcaccaccggc
K L P V P W P T L V T T L T W G V Q C F S R Y P D H M K
aagctgcccggtgccctggccaccctcgtagccaccctgacctggggcggtgcagtgcttcagccgctaccccgaccacatgaag
Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K
cagcacgacttcttcaagtcgcgcatgccgaagctacgtccaggagcgacccatcttcttcaaggacgacggcaactacaag
T R A E V K F E G D T L V N R I E L K G I D F K E D G N
acccgcgcgaggtgaagttcgagggcgacacccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaac
I L G G H K L E Y N Y I S H N V Y I T A D K Q K N G I K A
atcctggggcacaagctggagtacaactacatcacccacacgtctatatcacccgcgacagaagcagaagacggcatcaaggcc
N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G
aacttcaagatccgccacacatcgaggacggcagcgtgcagctgcgcgaccactaccagcagaacacccccatcgggcagcggc
P V L L P D N H Y L S T Q S A L S K D P N E K R D H M V
cccgtgctgtcgcccgacaaccactacgtgacacccagtcgcgcctgagcaaaagaccccaacgagaagcgcgcatcacatggtc
L L E F V T A A G I T L G M D E L Y K S G L E M S A E E
ctgctggagttcgtgaccgcccgcgggatcactctcggcatggacgagctgtacaagtcgggactcgagatcggtcgggcaggag
H S M S E V E M S P G V S D D G H S M S P G H S S G A P
cacagcatgtcggaggtggaatgagtcgccgggtgtcggacgatgggcaactccatgtcccctggtcactcgtcgggcgctccc
G G A D S P L P G Q Q S G I G D D G A G G V S G G V
ggtggcgcgactccctctgcccgtcagcagctcagatgtccgggatcggggatgatggagcgggtgactcctcgggcgggtc
S V K S D E E D D R F P I G I R E A V S Q V L N G Y D W
tcggtgaagtcgacgaggaagatgaccggttccccatcgccatccgcgagggcggtcagtcaggtgctgaacgggtacgactgg
T L V P M P V R V N S G S K S K P H V K R P M N A F M V
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W A Q A A R R K L A D Q Y P H L H N A E L S K T L G K L
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W R L L N E T D K R P F I E E A E R L R K Q H K K D Y P
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E Y K Y Q P R R R K N G K P G S S S E A D A H S E G E V
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E G K R E G A S R S G L G V G A D G S S A S G
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K P H I D F G N V D I G E I S H D V M A N M E P F D V N
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E F D Q Y L P P N G H P Q A S A T A S A G S A A P S Y T A
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Y G I S S A L A A A S G H S T A W L S K Q Q L P S Q Q H
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L G A D G G K T Q I K S E T H F P G D T A A S G S H V T
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Y T P L T L P H Y S A F P S L A S R A Q F A E Y A E H
tacacgcccgttaactgcgcactacagctccgccttcccctcgtggcgctcccgcgacaaattcgccgaatacggcagcac
Q A S G S Y Y A H S S Q T S G L Y S A F S Y M G P S Q R
caggcctcgggatcctactacgcccactccagccagacctcaggcctctactccgccttctcctacatgggccctcacagcgg
P L Y T A I P D P G S V P Q S H S P T H W E Q P V Y T T
cccctgtacacccgcatccggatccgggatccgtgcgcgagtcacacagccctacgcattgggagcagcccgatataccacaca
L S R P *
ctgtctcgaccgtgactagaactatagtgagtcgtattacgttagatccagacatgataagatacattgatgagtttggacaaac
cacaactagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgaaccattataagctgcaa
taaacaaagttaacaacaacaattgcattcattttatgtttcaggttcagggggaggtgtgggaggttttttaattcgcggccgc
ggc

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**Figure 4.12 Correct insertion of *sox10* into pTol2DctCFP to create pTol2DctCFPsox10**

Complete sequence, with translation above, between the start of the *CFP* sequence and the end of the *sox10* fragment. Junctions between *sox10* and the other components of the plasmid have been sequenced and verified using primers S22, BS1, seqCFP and S19. Purple - *ECFP* fragment from ECFP plasmid, AgeI-BspEI, Bright blue - *ECFP-sox10* ATG, Green - BspEI site used to clone *ECFP* into, Red - XhoI site used to clone *sox10* into, Orange - *Sox10* ATG, Dark blue - *Sox10* coding sequence, Green - *ECFP-sox10* STOP codon, Black - CS2 sequence from end of *sox10* to SfoI.





**Figure 4.13 pTol2DctCFP and pTol2DctCFPsox10**

A. Diagram of final pTol2DctCFP plasmid. Indicated are the restriction sites used for cloning.

B. Diagram of final pTol2DctCFPsox10 plasmid. Indicated are the restriction sites used for cloning and the primer binding sites for the primers used either during cloning or sequencing of the final plasmid. TYR1 is at 5437, seqCFP at 10080, S19 at 10800, S22 at 10890 and BS1 binds at 844.

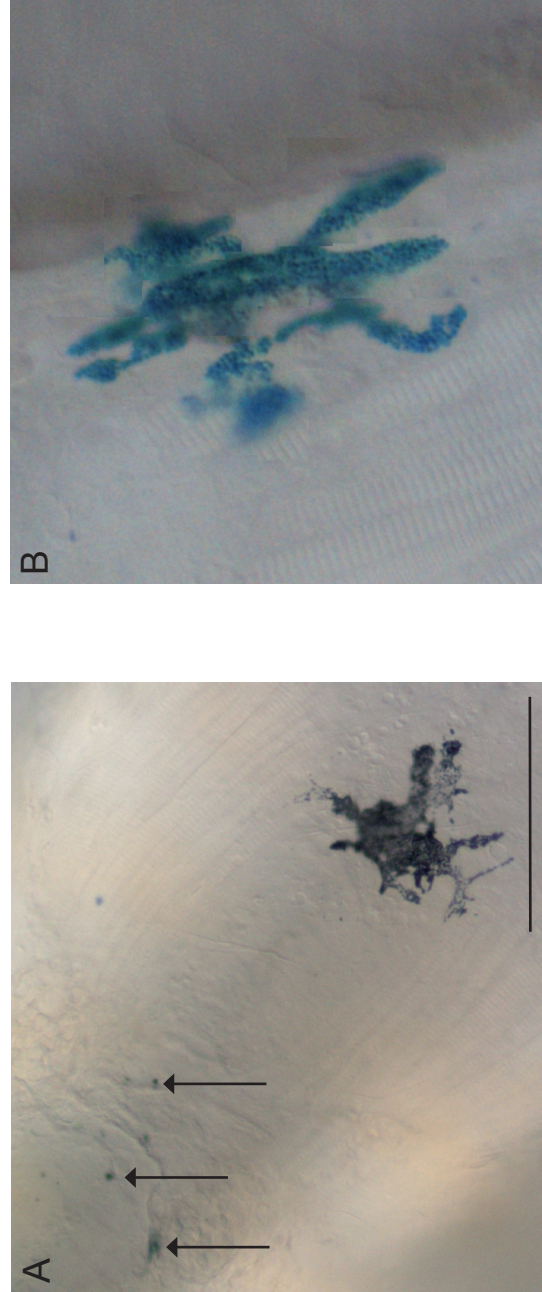
### 4.2.3 Generating Transgenics

#### 4.2.3i Testing Functionality of CFP-*sox10*

Prior to generating germline transgenics we had to test whether the Sox10 protein would still function when fused to CFP. We did not anticipate that the CFP would have any effect upon the functionality of Sox10 since previous transgenics in the lab had placed GFP upstream of *sox10* whilst retaining Sox10 function (J. Dutton, personal communication). However, it was still necessary to test our construct to confirm this. Previously, pigment cells in a *sox10* mutant have been rescued by injection of a plasmid containing *sox10* into one cell stage embryos (Dutton et al. 2001). In this case *sox10* was under the control of a heatshock promoter which was used to drive Sox10 expression at 10-12 hpf and 22-24 hpf to rescue melanophores. To test our constructs we performed a modified version of this experiment. We injected either pTol2DctCFPsox10 or pTol2DctCFP (as a control) at concentrations of 25ng/μl, together with transposase RNA at 25ng/μl, into one cell stage embryos from an incross of adults heterozygous for *sox10*<sup>l3</sup> and looked for rescue of pigment cells.

Embryos were injected and grown to 27 hpf. To confirm that the constructs were working successfully we checked that at least some embryos from each batch were expressing CFP at this stage. However, we grew and scored all fish, whether positive for CFP or not. This ensured that we did not miss embryos which might still go on to have rescued cells but which might be expressing CFP at lower levels or at a later stage than we screened them. All the embryos were grown to 72 hpf when the mutants could easily be selected by their severe reduction in pigment cells. Any escaper/rescued pigment cells were then scored. We expected that we might see rescue of any or all pigment cells so we looked for xanthophore and iridophore rescue as well as melanophore rescue.

*sox10*<sup>l3</sup> embryos lack properly formed neural crest-derived melanophores altogether and have only small, punctuate residual melanised cells by 72 hpf. We therefore knew that any melanophores we saw at this stage that were not small and punctuate could be described as rescued cells. Out of 221 mutant fish injected with the control plasmid pTol2DctCFP no fish showed any melanophores or melanophore-like cells. Out of 273 mutant fish injected with pTol2DctCFPsox10 three fish had a single melanophore and one had two (Figure 4.14). As we demonstrated in Chapter 3, *dct* is expressed in the neural crest of these mutants, but only in a limited number of cells and at very low levels. Since our construct uses a *Dct* promoter to drive expression of CFP-*sox10* we expected that only very few cells will express CFP-Sox10 and so only a few cells will be rescued. This is in contrast to the experiments from Dutton et al. (2001) where many more cells in the neural crest can be expected to express Sox10



**Figure 4.14 pTol2DctCFPsox10 produces functional CFP-Sox10 protein**

A. Bottom right, a rescued melanophore in a *sox10*<sup>3</sup> mutant injected with pTol2DctCFPsox10. Compare with residual pigment (arrows) characteristic even of uninjected *sox10*<sup>3</sup> fish. Scale bar 50μm.

B. A rescued xanthophore in a *sox10*<sup>3</sup> mutant injected with pTol2DctCFPsox10. Note xanthophores will often adopt a blue colour rather than yellow in embryos reared in embryo medium containing methylene blue.

and so the potential to rescue cells is greater. We considered the melanophores seen in fish injected with pTol2DctCFPsox10 to be cells rescued as a result of expression of CFP-Sox10 in these cells.

Xanthophores are not present in 72 hpf *sox10*<sup>-/-</sup> fish. Some xanthophores do form earlier in development but they have abnormal morphology and by 35-45 hpf they have died (Dutton et al. 2001). Since xanthophores also stain blue with methylene blue (Le Guyader and Jesuthasan 2002), we looked for blue/green cells of an appropriate morphology and location to be xanthophores. Again, out of 221 mutant fish injected with pTol2DctCFP none had any visible xanthophores. Out of 273 mutant fish injected with pTol2DctCFPsox10, one had a single fully developed xanthophore (Figure 4.14). We also saw xanthophores in two other embryos (one and two cells respectively) during our preliminary experiments. Again, despite the small sample size, in the absence of any xanthophores in the control embryos and due to the limitations of the experiment discussed above we consider these to be rescued cells.

The case for iridophores is more complex. Even untreated *sox10*<sup>-/-</sup> fish have some escaper iridophores (Kelsh and Eisen 2000) so instead of looking for any rescued cells at all we were in fact looking for an increase in iridophores over and above the normal escaper cells. Iridophores were counted in each fish, whether they were injected with pTol2DctCFP or pTol2DctCFPsox10. The data did not show a normal distribution so they were analysed using a Mann Whitney U test to compare the medians of the two sets of data. This test showed that the medians of the two data sets differed significantly with  $p < 0.0001$ . Returning to look at the actual data, it is possible to see that there is a decreased proportion of fish in the experimental group that have no iridophores at all. There is an increased number of fish in experimental group as compared to the control group which have one, two or three or more iridophores (Table 4.02). We interpret this as showing that our experimental plasmid has the effect of altering the median number of iridophores compared to the control plasmid and that this is as a result of increasing the number of escaper iridophores in *sox10*<sup>-/-</sup> embryos; in short, that pTol2DctCFPsox10 can rescue iridophores in *sox10*<sup>-/-</sup> embryos.

Therefore, we were satisfied that we had shown rescue of all pigment cell types in *sox10*<sup>-/-</sup> fish by injecting them with pTol2DctCFPsox10. This indicated to us that our CFP-Sox10 fusion would be likely to function in our germline transgenics.

#### **4.2.3ii Generating *Tg(Dct;CFP)* and *Tg(Dct;CFP-sox10)***

Having established that our pTol2DctCFPsox10 plasmid produced a CFP-Sox10 fusion that could function in transient transgenics (and, incidentally, that our pTol2DctCFP

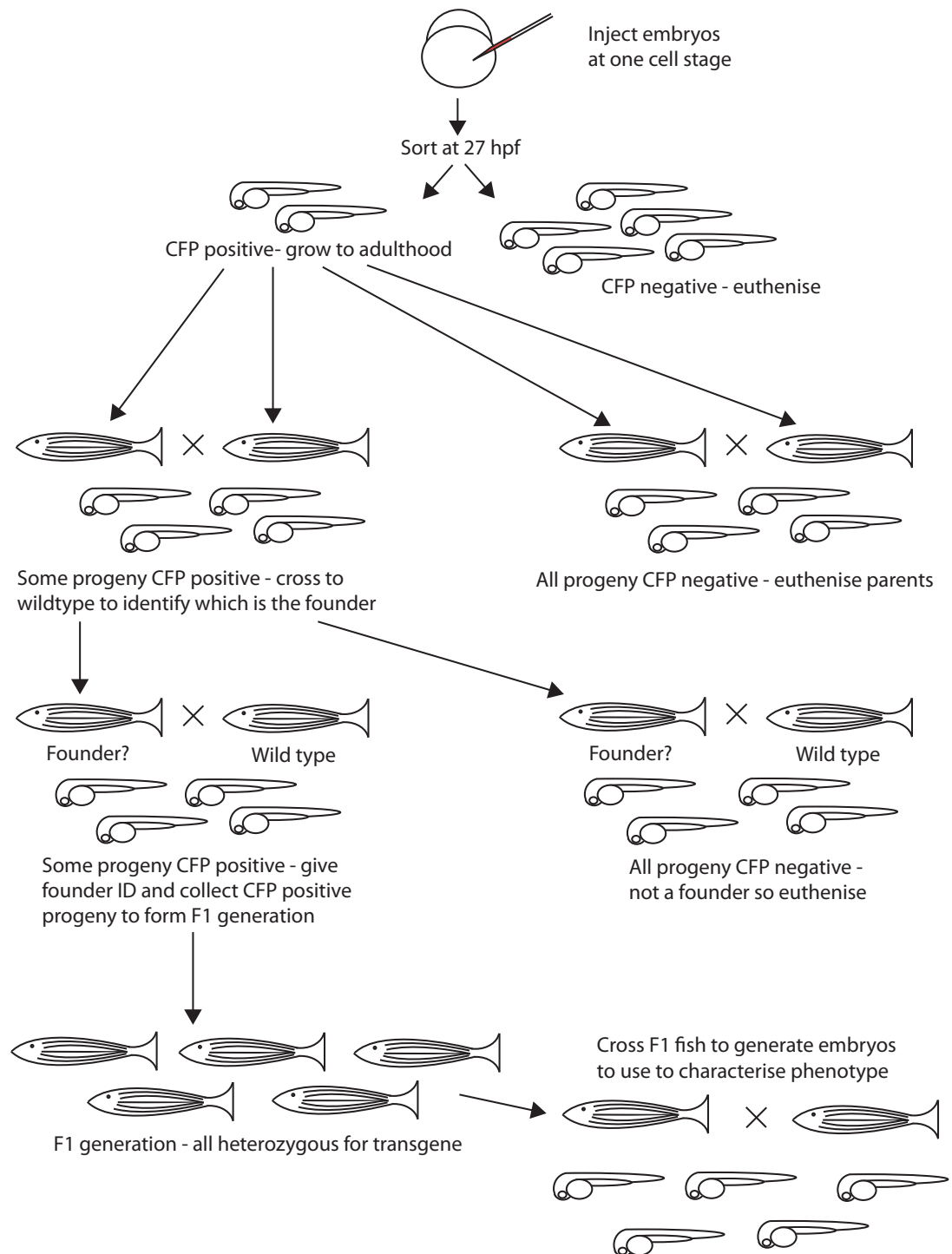
Table 4.02 Comparing the number of iridophores present in *sox10*<sup>3</sup> fish that had either been injected with pTol2DctCFP or with pTol2DctCFPsox10

Number of iridophores per fish	pTolDctCFP injected Number of embryos	pTol2DctCFP injected Percentage of embryos	pTol2DctCFPsox10 injected Number of embryos	pTol2DctCFPsox10 injected Percentage of embryos
0	165	75	143	52.4
1	36	16	63	23.1
2	13	6	44	16.1
3 - 5	7	3	23	8.4
Total	221	100	273	100

The medians of the two sets of data differ significantly,  $p < 0.0001$ . In the experimental injected set, there is a lower percentage of embryos with no iridophores and a higher percentage in each of the groups where embryos have 1, 2 or 3-5 iridophores.

plasmid caused cells to make functional CFP protein) we continued to generate germline transgenics. For a schematic of how this was achieved see Figure 4.15. To generate transgenic lines each plasmid was injected into eggs at the one cell stage together with transposase RNA at 25ng/ $\mu$ l. pTol2DctCFP was injected at a concentration of 50ng/ $\mu$ l, pTol2DctCFPsox10 at 25ng/ $\mu$ l. These fish were screened at approximately 27 hpf for expression of CFP. Those showing CFP expression were selected and grown to adulthood; we reasoned that embryos expressing CFP at levels high enough to observe may be more likely to have incorporated the transgene into their genome and may be more likely to show germline transmission than those that did not show CFP expression. Similarly, it should be noted that to maximise the likelihood of the transgene integrating into the genome it is desirable to ensure that there is a high death rate among injected embryos, since if DNA is injected at the maximum concentration that the embryos can tolerate it maximises the chances of transgene incorporation into the genome. Amongst approximately 4500 fish injected with pTol2DctCFP, we observed a death rate of 65.2 %. Of the fish that survived, 21 % showed CFP expression and were grown to adulthood. 93 fish, named Tg(Dct;CFP), survived to adulthood. Amongst approximately 3500 fish injected with pTol2DctCFPsox10, we observed a death rate of 37.7 %. 12.8 % of surviving fish showed CFP expression and were grown to adulthood. Approximately 45 fish, named Tg(Dct;CFP-sox10), survived to adulthood. These potential founder fish were then screened to determine whether or not they were able to transmit the transgene through their germline. Fish were incrossed initially to quickly screen as many fish as possible. At least 50 embryos from each of these initial crosses were observed at approximately 27 hpf. It has been found that when using the Tol2 system 27 – 100 % of F1 embryos are positive for the transgene in question (Kawakami et al. 2004). If this is the case in our experiment then a founder should not transmit at such a low rate that we will miss it in our screening. If none of the F1 embryos scored had any CFP expression the parents were deemed not to be transmitting through their germline and were euthanised. If, however, some of the embryos did display CFP expression we knew that either or both of the parents were able to transmit the transgene through their germline. These fish were then outcrossed to wild type fish to find which parent was transmitting the transgene. Again, any fish that didn't transmit the transgene were euthanised. Once a fish had been identified as transmitting through the germline it was placed in a separate tank and given an ID, a name beginning with 'C' for Tg(Dct;CFP) founders and a name beginning with 'S' for Tg(Dct;CFP-sox10) founders, e.g. 'Charlie' or 'Sam'. In this way, all of the Tg(Dct;CFP) fish and approximately 32 of the Tg(Dct;CFP-sox10) fish were screened. 14 Tg(Dct;CFP) founder fish and 14 Tg(Dct;CFP-sox10) founder fish were identified. We aimed to collect 100 CFP positive progeny from each founder fish (outcrossed to AB) and these now formed the F1 generation of each new line (14 Tg(Dct;CFP) and





**Figure 4.15 Generating transgenics**

Embryos are first injected at the one cell stage with our constructs, together with transposase RNA. The embryos and their progeny then undergo a series of selection procedures to find those that are CFP positive and then those that are able to transmit our transgene to their progeny. This method was used to generate two transgenic lines, Tg(Dct;CFP) and Tg(Dct;CFP-sox10).

11 Tg(Dct;CFP-sox10) lines). These fish should all be heterozygous for the transgene and were grown to adulthood for use in further experiments.

#### 4.2.4 Characterisation of Tg(Dct;CFP)

Prior to choosing which lines to maintain and use for further experiments we performed a quick screen for CFP expression in each line. Embryos were scored at 27 hpf and 48 hpf under a Nikon Eclipse 300. This allowed us to ensure that the expression patterns were as we expected and to note any ectopic expression. For these initial observations F1 lines were incrossed and their embryos observed at 27 hpf and 48 hpf. This also checks for any recessive effects of the transgene. We did not notice any recessive effects of our transgene, for example death rates and rates of malformation did not appear increased.

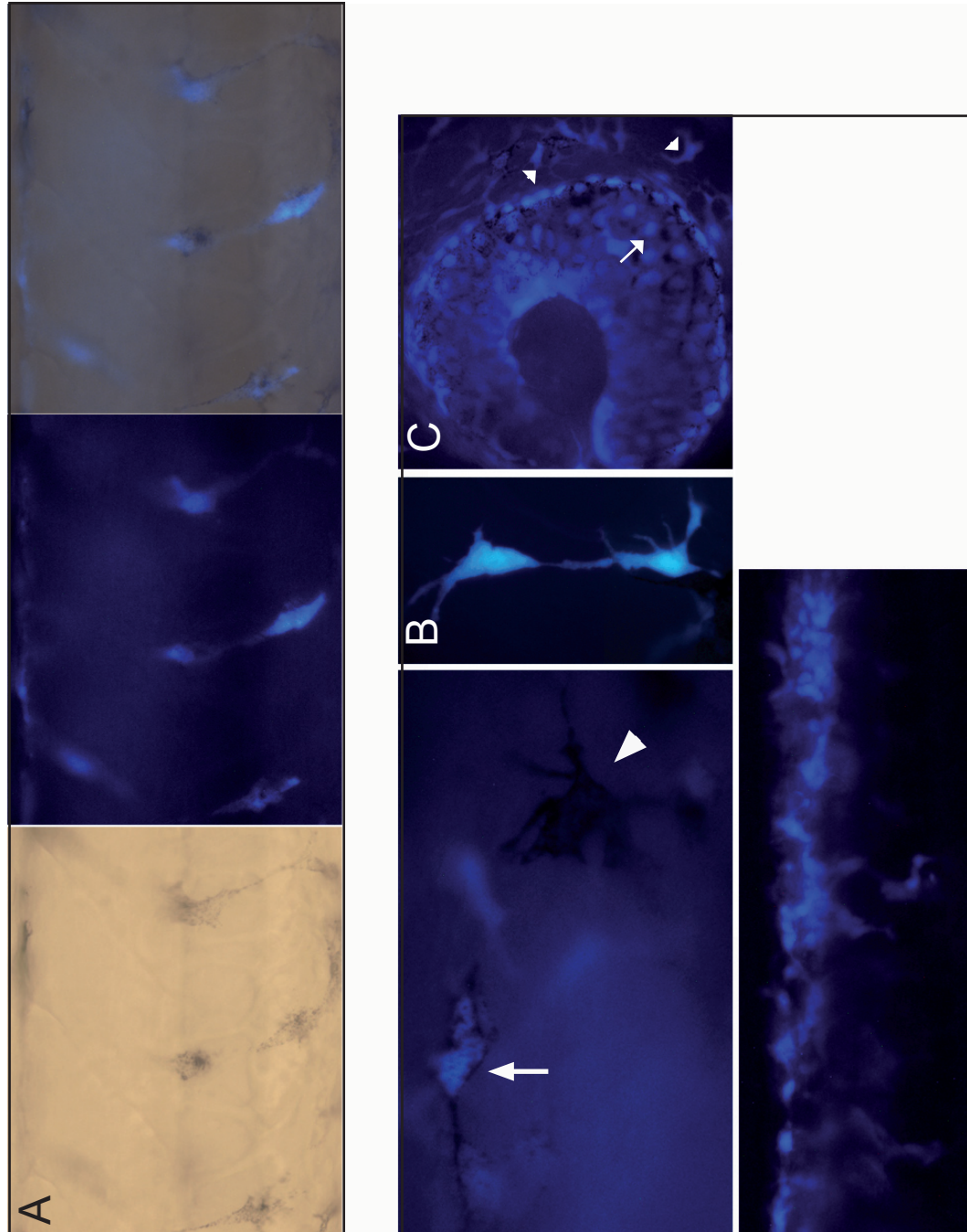
Although the levels of CFP expression were variable between lines, Tg(Dct;CFP) lines generally had fairly weak CFP expression. It was useful that we had performed incrosses of probable heterozygotes and therefore noticeably brighter individuals were present, which we assumed to be homozygous carriers of the transgene, that were easier to observe. It did however, mean that where we were forced to outcross the F1 fish (due to unfavourable sex ratios in particular lines) expression was invariably even weaker. These lines were more difficult to analyse. Of four lines which were outcrossed no CFP expression was seen at any stage in two, and only at 27 hpf in the remaining two. It may be that this apparent loss of transgene expression was in fact due to human error in the process of sorting these embryos. Alternatively it may represent a real lack of expression at one or both stages in these lines or that it is too weak and we simply cannot detect it (since we are only observing heterozygotes). Of the lines which we were able to incross all had broadly the same expression pattern. A summary of the expression patterns seen is in Table 4.03. At 27 hpf, expression was seen in the PRE of the eye, in cells in a premigratory neural crest position and in neural crest cells migrating on the medial and lateral pathways, as well as in pigmented melanophores. However, some pigmented melanophores at this stage did not express CFP (Figure 4.16). It was also noted that several lines appear to have stronger CFP expression more posteriorly, i.e. in younger crest cells and less CFP expression in migrating/older crest. These observations suggest that expression from the promoter is strongest at earlier stages and that it may be downregulated at later stages. Some ectopic expression was also seen at this stage. Two lines had CFP expression in the CNS together with cells expressing CFP in a melanoblasts/melanophore pattern (Cuthbert and Clint). One line, Chris, did not have CFP expression in melanophores but instead had expression in xanthophores as identified by their characteristic spindly shape and by their location only on the lateral pathway (Figure 4.17).

Table 4.03 CFP expression pattern in Tg(Dct;CFP) lines

Line	27 hpf		Pattern consistent with melanoblasts/ melanophores?	Ectopic expression	48hpf	
	Brightness				Melanophores	Ectopic expression
Colin	***	Yes			Yes	
Charlie	***	Yes - stronger posteriorly			Yes	
Cameron	**	Yes			Yes	
Clive	*	Yes			Yes	Some heart expression
Cuthbert	***	Yes - Less in migrating cells		Ventral CNS	Yes	Spinal cord
Conrad	**	Yes			Yes	
Caesar	*	Yes - Only in v few pigmented cells			Yes	
Chris	***	No		Xanthophores	Yes	Xanthophores
Clint	***	Yes - Not in older crest		Dorsal CNS	Yes	Brain, spinal cord
Carlos	*	Yes			Yes	
Casper *	*	Yes - In few cells and very variable			No	
Cactus *	*	Yes			No	
Cecil *		Nothing in 12 embryos examined			No	Spinal cord
Caliste *		Nothing in 12 embryos examined			No	

Most lines were analysed by generating F2 embryos from an F1 incross, except for those marked with '\*' where F1 fish were outcrossed to wildtype fish. 4-12 embryos were examined from each line at both 27 hpf and 48 hpf; at 48 hpf, embryos had been PTU treated from 24 hpf. At 27 hpf, embryos were scored as having a pattern consistent with melanoblasts/melanophores if they had CFP expression in the eye, in a premigratory neural crest position, in cells migrating on both the medial and lateral pathways or if expression could be seen in pigmented melanophores. The lines varied in the strength of CFP signal, which we have attempted to represent in the table. Some lines showed ectopic/unexpected expression patterns, note particularly Chris where CFP expression was seen in xanthophores, i.e. in cells only on the lateral pathway with a very characteristic spindly morphology. All expression seen at 48 hpf was very weak. We scored fish as having expression in melanophores where we could see CFP expression in all stripes (particularly the lateral stripe) and where this expression was not visible using different wavelengths of light and did not photobleach.

**Figure 4.16 CFP expression in Tg(Dct;CFP) lines is consistent with sites of endogenous *dct* expression**



#### Top panel

A-C. CFP expression in pigmented cells migrating on the lateral pathway in a 27 hpf F2 Clive embryo. A. DIC image. B. Fluorescent image. C. Merge of A and B, note close overlap of pigment and fluorescence.

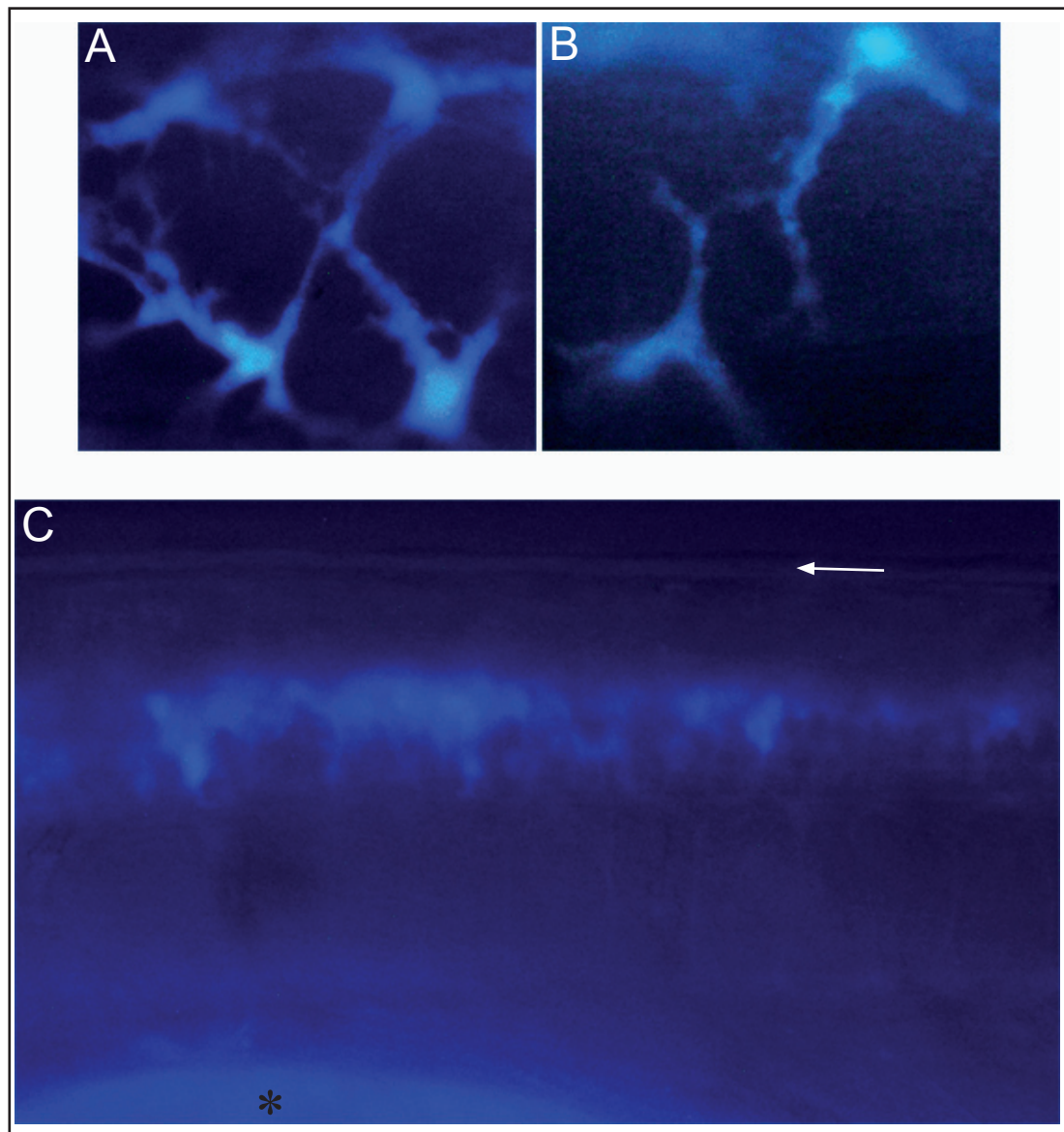
#### Bottom panel

A. Two melanophores in the head of a 27 hpf F2 Caesar embryo. Note that one melanophore has CFP expression (arrow) whilst the other does not (arrowhead).

B. Two CFP positive, unpigmented cells in the head of a 27 hpf F2 Clint embryo. These may be unpigmented melanoblasts or another cell type.

C. The eye of a 27 hpf F2 Colin embryo showing CFP expression in tessellating melanophores of the PRE (arrow) as well as CFP positive cells outside of the eye (arrowheads), some of which are pigmented whilst others are not. D. Premigratory cells in the posterior tail of a 27 hpf F2 Charlie embryo.





**Figure 4.17 Examples of ectopic CFP expression seen in Tg(Dct;CFP) lines**

A and B. CFP expression in cells on the lateral migration pathway of F2 Chris embryos at 27 hpf and 48 hpf respectively. CFP expression was only seen in cells in a premigratory position and on the lateral pathway (not on the medial pathway). This, along with the characteristic spindly morphology of the cells, suggests that they are xanthophores.

C. CFP expression in dorsal cells of the spinal cord of an F2 Clint embryo at 27 hpf. Cells are present through the whole spinal cord from left to right. Anterior to left, posterior to right, dorsal top, ventral bottom. Dorsal edge of embryo can just be seen (arrow) as can curve of the autofluorescent yolk sac (bottom left, \*).

When examined at 48 hpf embryos were treated with PTU from 24 hpf to prevent melanin from blocking the fluorescence. Most of the Tg(Dct;CFP) lines had CFP expression in cells of a position and morphology consistent with their being melanophores. The exceptions were the two outcrossed lines which had had expression of CFP at 27 hpf but no longer had visible CFP at 48 hpf. Expression at 48 hpf was generally even weaker than at 27 hpf supporting the suggestion that our transgene may be being turned off by this stage. Cells could be seen in the eye, dorsal and ventral stripes and more importantly in the lateral stripe. This latter fact, together with the morphology of the cells, was particularly important to differentiate the cells from auto-fluorescent iridophores, which are not present in the lateral stripe. There were cells observed in all of the stripes which were only fluorescent using the CFP filter and not any of the other filters (i.e. the cells were not auto-fluorescent and so not iridophores/xanthophores). In addition, the cells did not photobleach after prolonged UV exposure (as autofluorescence from xanthophores would). The ectopic expression seen in some of the lines at 27 hpf persisted to 48 hpf with the addition of heart expression in some Clive embryos. This heart expression was not always present in embryos which had CFP expression in melanophores.

CFP expression was also observed in all of the Tg(Dct;CFP) lines at earlier stages; 20 hpf and 24 hpf. At 20 hpf CFP was present in the developing eye only. By 24 hpf it was also seen in neural crest-like cells in the anterior trunk. Their position and morphology was characteristic of early migrating neural crest cells as can be identified by ISH for *dct*. Whilst this pattern of expression mimics that of *dct* when it is first switched on, the timing is delayed by approximately 5 hours (Kelsh, Schmid and Eisen 2000).

By judging the lines for brightness of CFP expression, how faithfully the *dct* expression pattern was recapitulated and the number of fish in the F1 generation, we selected three Tg(Dct;CFP) lines to continue to work with – Charlie, Cuthbert and Cameron.

#### **4.2.5 Characterisation of Tg(Dct;CFP-sox10)**

No obvious melanophore phenotype had been noted in any of the Tg(Dct;CFP-sox10) lines examined, i.e. there was no dramatic loss of melanophores or striking delay in their differentiation. In addition, a brief exploration of melanophore number at 30 hpf and at 46 hpf revealed no significant difference between the mean number of melanophores in three Tg(Dct;CFP) lines and three Tg(Dct;CFP-sox10) lines at these stages (data not shown). We examined F2 embryos from eight out of eleven Tg(Dct;CFP-sox10) lines and found that none of those examined showed any CFP expression at all at any stage. We did not examine the three remaining lines due

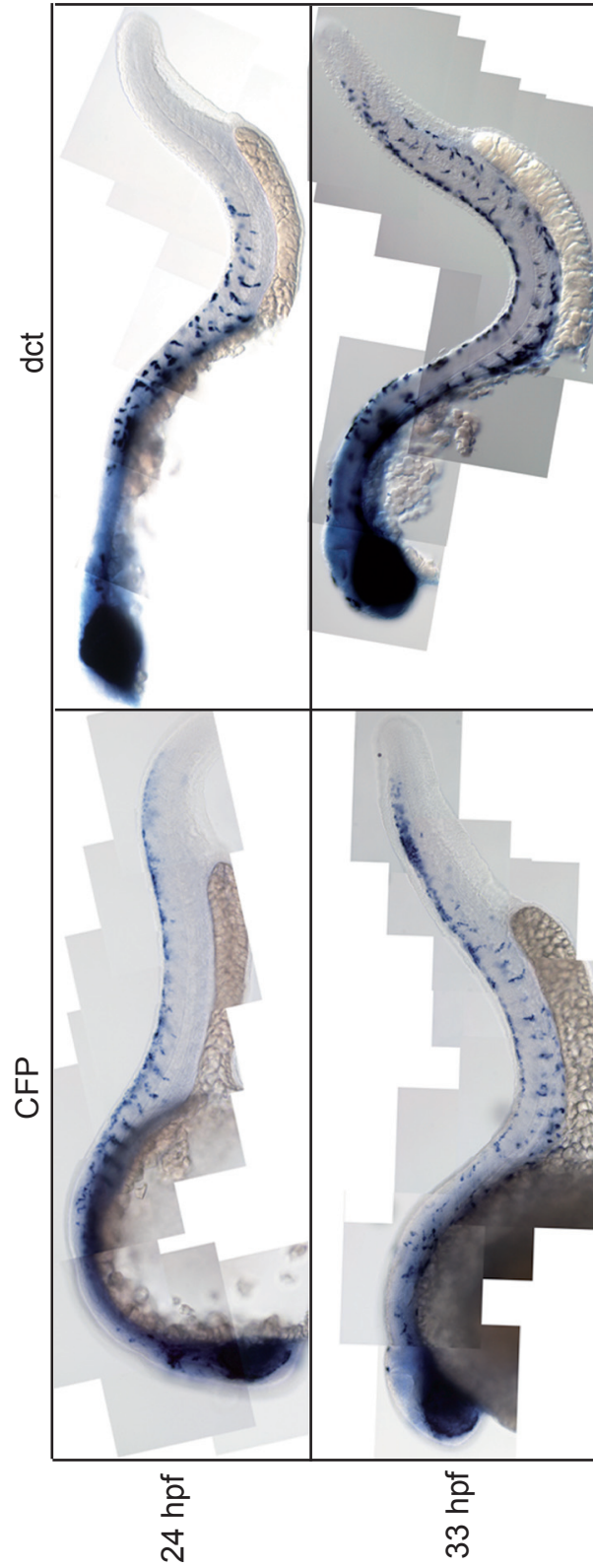


to difficulties in obtaining F2 embryos from them. We hypothesised that a lack of functional CFP-Sox10 fusion protein would explain a lack of melanophore phenotype in these embryos. Since we could not find any CFP expression in our Tg(Dct;CFP-sox10) lines we needed to investigate by other means the functionality of the Dct;CFP-sox10 transgene. The first possibility to test was that the transgene was not being transcribed and so could not be translated into protein. We generated an ISH probe for CFP which we used on F2 embryos from Tg(Dct;CFP-sox10) and Tg(Dct;CFP) lines at different stages to look for transgene transcript. The second possibility was that the transgene had been removed from the genome or that the sequence had been disrupted. We looked to confirm that our F1 generation fish were carriers of the transgene by genotyping F1 fish from the Sam, Sebastian and Simon lines and to confirm that the transgene persisted in the F2 generation by also genotyping F2 embryos.

#### **4.2.5i Detection of CFP RNA by In Situ Hybridisation**

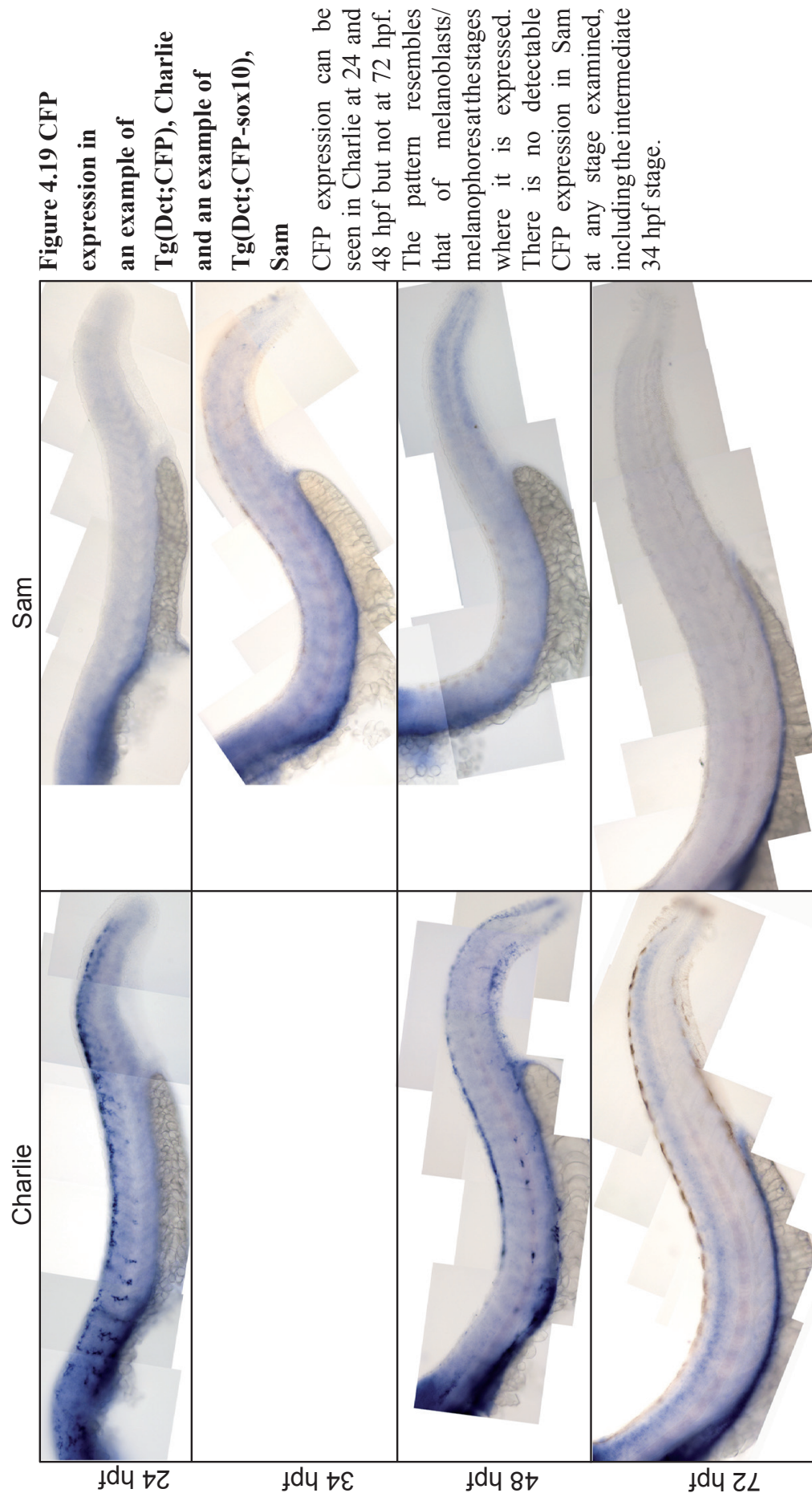
We generated a plasmid containing *ECFP* to make a *CFP* ISH probe. This allowed us to look at *CFP* expression at the RNA level. Comparing the pattern of *CFP* expression to that of *dct* in Tg(Dct;CFP) embryos, we can see that this pattern is generally consistent with CFP being expressed, as expected, in cells which express *dct* in this line (Figure 4.18). However, *CFP* appears to be expressed in younger, more posterior, cells than *dct* at 24 hpf. The expression patterns observed in different embryos varied but *CFP* did seem to be expressed more weakly than *dct*, particularly in older cells at 33 hpf suggesting that it is being switched off at this time. This is consistent with our observations of CFP protein in live embryos.

We looked for *CFP* expression in our Tg(Dct;CFP-sox10) lines. Charlie was used as a control line to compare against Sam, Sebastian and Simon. F2 embryos from each of these lines were fixed at 24 hpf, 48 hpf and 72 hpf with an additional timepoint at 34 hpf for the Tg(Dct;CFP-sox10) lines. *CFP* expression was seen at 24 hpf and 48 hpf in F2 Charlie embryos in a pattern resembling that seen for CFP protein in this line. At 24 hpf *CFP* positive cells could be seen both in a premigratory position and in migrating cells on the medial and lateral pathways. At 48 hpf *CFP* could be seen in a pattern resembling that of melanophores at this stage, in dorsal, lateral and ventral stripes (Figure 4.19). At both stages *CFP* expression could also be seen in the eye (data not shown). By 72 hpf *CFP* expression was no longer detectable in Tg(Dct;CFP) embryos. However, no detectable expression of *CFP* was seen at any stage in any of the Tg(Dct;CFP-sox10) lines (Figure 4.19). We concluded that our transgene transcript is not transcribed or maintained at levels detectable by in situ hybridisation in Tg(Dct;CFP-sox10) F2 embryos. It is likely that this explains the lack of CFP-Sox10 protein detectable by fluorescence microscopy in these embryos.



**Figure 4.18 A comparison of *dct* and CFP expression patterns in F2 Charlie at 24 and 33 hpf**

At 24 hpf, the expression patterns of *dct* and CFP are similar. Here however, CFP seems to be on earlier than *dct*, i.e. in more posterior cells. CFP expression is also weaker than *dct* expression. By 33 hpf, the two expression patterns are more alike showing expression in cells in the dorsal stripe as well as in migrating cells. CFP is expressed more in premigratory neural crest than *dct* though and does appear to be more weakly expressed (these ISHs were developed concurrently), particularly anteriorly where it may be being turned off. It should be noted that the CFP expression patterns were variable between embryos.

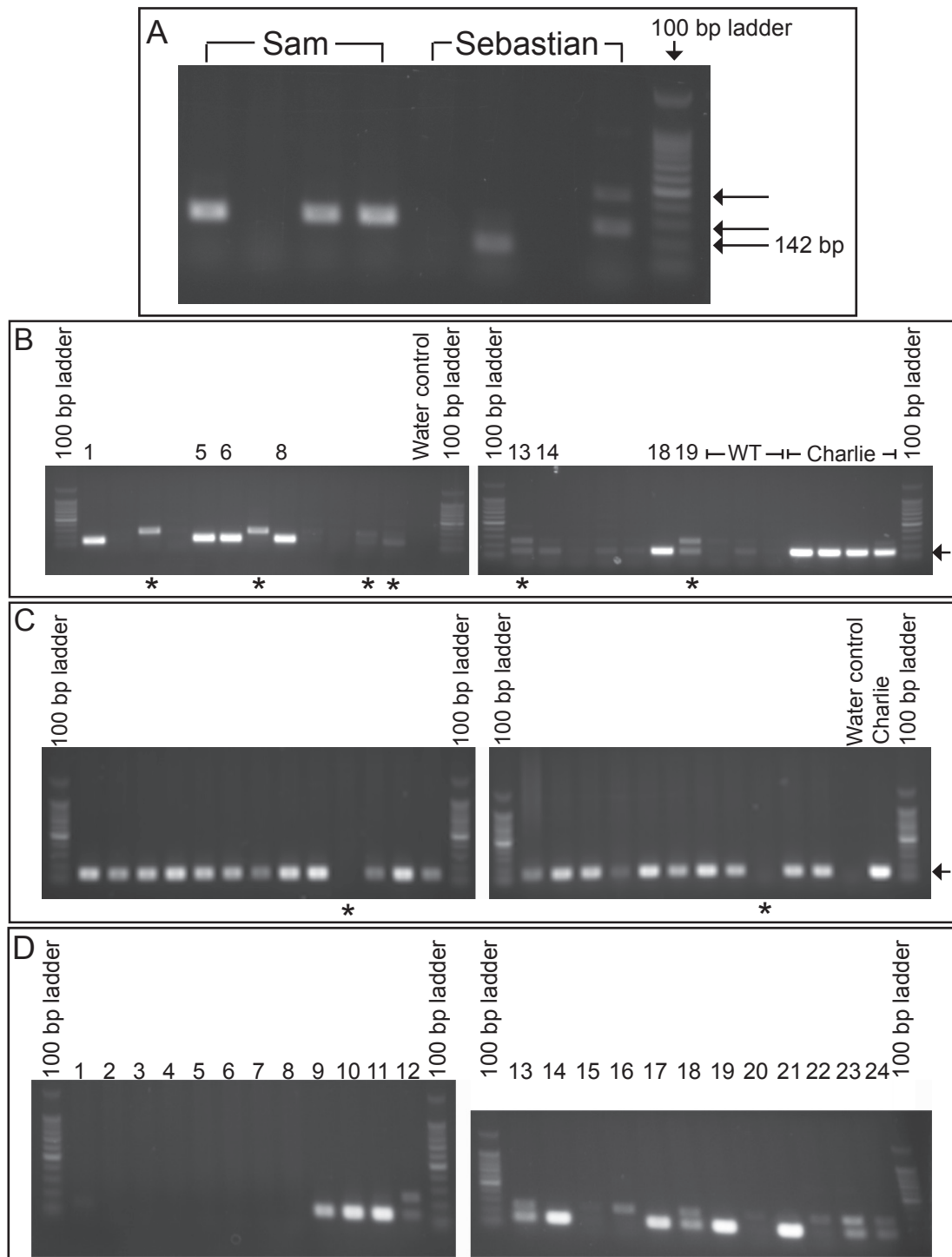


#### 4.2.5ii Genotyping of F1 Parents and F2 Progeny

Another hypothesis to explain the lack of transgene transcript is that our transgene was lost from or disrupted in the genome of F1 fish. We now tested whether our F1 Tg(Dct;CFP-sox10) fish had our transgene incorporated into their genomes. Primers were designed that would amplify a 142 bp fragment of *CFP* (see Appendix for sequences). Fin clips were taken from each of the fish of the F1 generation of Sam, Sebastian and Simon plus five fish from the F1 generation of Charlie as a positive control. Genomic DNA was extracted from these samples and used in PCR. Initial experiments looking at the first five fish from each line revealed that our primers produced three different sized bands (including a 140 bp band) in different fish and some fish showed no amplification at all (see Figure 4.20 A). We cloned each of these bands to identify what we had amplified. The 142 bp band proved to be the *CFP* sequence that we were expecting. The two larger bands contained sequence from the pT2KXIG Tol2 plasmid into which our original construct had been built which had been amplified due to weak sequence similarities with our primers. Why plasmid sequence was incorporated into the genome instead of or as well as our transgene is unclear.

At least one fish from the Sebastian line had the appropriate 142 bp *CFP* band (Figure 4.20 A). We wanted to identify this fish and determine whether it was able to transmit the transgene to the F2 generation. If it was not able to do this then this would explain why we do not see detectable transgene expression in the F2 generation. We went back to the F1 Sebastian generation and reclipped each fish, this time keeping each fish separately. We genotyped these samples and found that again there was the same variety of PCR product sizes (Figure 4.20 B). Some fish did not produce any product, either because they were not transgenic or because the PCR did not work on these samples. As is visible in Figure 4.20, two of the three wild type negative controls had very faint bands which we believe to be contamination of the DNA from repeated use. Our water control was negative however so we selected the eight fish with the strongest bands in the 142 bp position for further experiments. We incrossed one female with two males and outcrossed the remaining fish with wild types. Of the four clutches produced (including one from the incross) no embryos had visible CFP expression between 24 hpf and 30 hpf. We genotyped 24 embryos from three clutches (not including the incross) and found that many of the embryos did contain our transgene as well as bands representing vector insertions (Figure 4.20 C and D). Interestingly, the ratios for each mating did not appear to conform to Mendelian ratios expected from a mating of heterozygous fish and the banding patterns were often variable within a clutch.

Thus, we have established that our F1 generation transgenics are transgenic and that the transgene is passed on to the F2 generation. However, CFP protein or RNA expression



**Figure 4.20 F1 and F2 Tg(Dct;CFP-sox10) fish have the transgene**

A. Five F1 fish from Sam, Simon and Sebastian were genotyped using primers for CFP. Four from Sam and Sebastian are shown here. One fish has a band in the appropriate place for the CFP product, 142 bp. Others had additional bands which were cloned for analysis.

B. All 19 F1 Sebastians were genotyped. Several had the correct band whilst others also had one or two larger bands one of which is similar to that in A (\*).

C. F2 embryos from an incross of fish 1, 5 and 6 from B. All but two (\*) out of 24 had the 142 bp band.

D. F2 embryos from an outcross of fish 19 from B. 2-8 negative, 1, 9-11, 14, 17, 19 and 21 have the 142 bp band, 15, 16, 20 and 22 have a larger band, the rest have both.



is not detectable in these fish. This explains the lack of any obvious melanophore phenotype in our Tg(Dct;CFP-sox10) lines and makes it unlikely that there will be a more subtle phenotype.

## 4.3 Discussion

### 4.3.1 Sox10 is Downregulated in Differentiating Melanophores

In Chapter 3 we demonstrated that in the absence of functional Sox10, melanophore differentiation genes are derepressed. This implies that in normal development Sox10 acts to inhibit melanophore differentiation by repressing these same genes. If this is the case then Sox10 would eventually have to be downregulated in melanophores to allow them to differentiate. Thus, we looked at the expression both of Sox10 protein and *sox10* mRNA in melanophores as they pigment. If Sox10 is able to inhibit melanophore differentiation as we predict then it should not be expressed in differentiating cells. We found that Sox10 and *sox10* are both expressed in pigmenting cells but that they are downregulated over time. This provides further data in support of our model for the role of Sox10 in melanophore development.

It is interesting to note that Sox10 and *sox10* are still expressed in some pigmented cells; if Sox10 strictly inhibits differentiation then it might not be expected to be present in differentiating cells. However, Kim et al. (2003) report that levels of Sox10 are important in its various roles in sympathetic neuron differentiation so that whilst only comparatively low levels of Sox10 expression are sufficient for activation of *Mash1* and *Phox2b* (these genes are still expressed in heterozygous *Sox10* mutants), much higher levels are required to inhibit expression of *Phox2a* (expression of this gene is derepressed even in heterozygous *Sox10* mutants). Similarly, it may be the case in melanophores that only low levels of Sox10 are required for *mitfa* expression but higher levels are required to inhibit expression of melanophore differentiation genes. Thus, Sox10 could still be expressed in pigmenting cells but at levels not high enough to inhibit differentiation.

Our results demonstrating downregulation of *sox10* show a gradual loss of the mRNA from pigmented melanophores after 30 hpf. There is a possibility that the loss of Sox10 and *sox10* expression that we observe over time is due to the increasing amounts of melanin obscuring any signal that is present. We feel that this is unlikely as we observed two cells within one embryo which were pigmented to the same extent, one of which was Sox10 positive and one Sox10 negative thus confirming the presence of Sox10 negative melanophores (Figure 4.01). In addition, our results were consistent with previous work by Dutton et al. (2001) and Carney (2003). Dutton et al. (2001)



comment that at 36 hpf they are still able to see pigmented cells in the dorsal stripe expressing *sox10* whilst melanophores on the yolk are *sox10* negative. Dutton et al. (2001) also report a loss of *sox10* positive cells from the lateral pathway at 24 hpf and 30 hpf, timepoints where we have identified no *sox10* negative melanophores. However, they refer only to cells on the lateral pathway, whereas we have looked at premigratory cells and cells on the medial and lateral pathways. This suggests that there may be differences between different migratory populations that we would not have picked up. There may also be differences between our technique and that used in Dutton et al. (2001) which could explain the different results by, for example, giving increased sensitivity in our experiments so that we could identify cells that were expressing *sox10* even very weakly. It might be of interest in further experiments to score each melanophore according to its position as well as whether or not it is expressing Sox10 or *sox10* to enable us to make more detailed comments about different migratory populations of melanophores and the timing of loss of Sox10 or *sox10* from these cells.

Our data also support and extend previous work by Carney (2003) where a loss of GFP from pigmenting cells was observed in a transgenic line expressing GFP under the control of 4.9 kb of the *sox10* promoter. A rapid loss of GFP from melanophores is reported in these embryos but Carney (2003) reports that GFP is still present in a few melanophores at 4 dfp when we now know from our own work that Sox10 expression is lost by 54 hpf. We know that GFP shows good perdurance (Carney 2003) which may partly explain these differences. The dynamics of loss of Sox10 and GFP also differ in that our Sox10 graph follows a sigmoid curve but the GFP data followed an exponential decay curve. The difference between these data probably represents differences in the way that GFP and Sox10 proteins are turned over in the cell. Exponential decay, as for GFP, is consistent with loss of protein by simple degradation over time (Carney 2003). A sigmoid curve, as for Sox10, might suggest a more controlled degradation of the protein. We know that Sox10 can be SUMOylated (Girard and Goossens 2006) and we know that SUMOylation protects a protein from being ubiquitinated and marked for degradation (Hay 2005). This might be one means by which the stability of Sox10 and its rate of degradation could be controlled in the cell. The rate of loss of *sox10* mRNA is different again, the relationship between proportion of *sox10* positive cells and time being a linear one. This may reflect differences between turnover of mRNA and protein. It appears that in this case *sox10* mRNA is turned over more rapidly than the protein and protein levels only start to decrease once mRNA levels have dropped. We know that Sox10 protein in mouse cell culture can be shuttled between the nucleus and the cytoplasm (Rehberg et al. 2002). This could be a mechanism to allow for a slower initial rate of degradation of Sox10 compared to *sox10* since the protein does not

need to be degraded to be rendered non-functional. However, we found no evidence for cytoplasmic localisation of Sox10 in our own studies where, at the scale we could observe, localisation appeared to be nuclear.

In mouse, Sox10 expression is not downregulated but persists in neural crest cells as they migrate away from the neural tube and home to the skin (Osawa et al. 2005). There are several subsets of melanoblasts in mouse skin/hair follicles and these behave differently with respect to their expression of Sox10. Sox10 is expressed in all melanoblasts at embryonic stages. However, from postnatal day 2 Sox10 expression begins to be lost from melanoblasts as they become melanocyte stem cells. This seems to be important for the cells to maintain a stem cell fate. Other melanoblast/melanocyte populations maintain Sox10 expression at the same stage (Osawa et al. 2005). It is not clear what happens to Sox10 expression in these cells at later stages and late differentiation of mouse melanocytes as compared to zebrafish could mean that these later stages are more comparable to our studies. Results from Osawa et al. (2005) were obtained using a combination of in situ hybridisation, immunohistochemistry and single cell PCR from skin sections to describe the gene expression profiles of melanoblasts. However, the work is not exhaustive and it is particularly important to note that mice were not scored for Sox10 expression between E18.5 and P2 which leaves a gap in the data set of approximately five days. Other evidence from mouse melanoma cell lines also suggests that differentiated melanocytes still express Sox10, although of course these cells are by no means comparable to ‘normal’ melanocytes in vivo (Southard-Smith, Kos and Pavan 1998). Thus, in mouse it seems that Sox10 may be required in melanocytes as they differentiate and needs to be turned off in melanocyte stem cells. By contrast, we see Sox10 downregulation in cells as they differentiate and we have evidence that Sox10 promotes a stem cell-like state by inhibiting differentiation in zebrafish melanoblasts. This highlights the apparent differences between mouse and zebrafish, as discussed by Hou, Arnheiter and Pavan (2006), in the role of Sox10 in melanoblasts, melanocytes and melanophores. However, there is further evidence from cultured human melanocytes showing that, as in zebrafish, Sox10 is downregulated in these cells as they differentiate (Cook et al. 2005). Further careful and directed study in mouse and on human skin samples without culture with the aim of providing in vivo expression data for comparisons between organisms would be valuable.

### **4.3.2 Mixed Results from Generating Transgenics**

As we demonstrated in Chapter 3, Sox10 is able to inhibit expression of melanophore differentiation genes and we now know it is downregulated in differentiating melanophores. These two facts led us to hypothesise that it may be necessary for Sox10 to be downregulated to allow melanophores to differentiate. To test this hypothesis we

sought to generate two transgenic lines. One line would express CFP under the control of the mouse *Dct* promoter and act as a control for further experiments. The second line would express a CFP-Sox10 fusion under the control of the same promoter. In this way we would express Sox10 in melanophores at times later in development than it would normally be expressed. If our hypothesis, that Sox10 can inhibit melanophore differentiation and its downregulation is necessary for melanophore differentiation, is true then we would expect to see a cessation or delay in melanophore development in the latter transgenic line.

Generation of constructs for use in making each transgenic was successful; the plasmids were named pTol2DctCFP and pTol2DctCFPsox10. We were also able to successfully generate multiple lines (named Tg(Dct;CFP)) using the pTol2DctCFP plasmid. When examined in the F2 generation, many of these lines expressed CFP in their melanoblasts and melanophores as expected. However, there was some indication that expression of CFP was turned off at later stages. This is not comparable to the endogenous expression of *dct*, expression of which persists until at least 8 dpf (Kelsh et al. 2000). Nevertheless, these lines would provide a valuable control for our experimental transgenics and are also a useful tool in their own right. They could be used in any number of experiments where there is a need for melanoblasts to be marked separately from other neural crest derivatives. We know of another zebrafish line which expresses GFP in melanophores under the control of the Fugu *tyrp1* promoter (Zou et al. 2006). Our lines have an advantage over this line since they express CFP rather than GFP, this means they can be used in conjunction with other transgenic lines such as Tg(-7.2*sox10*:GFP) which mark all neural crest cells with GFP. This would enable researchers to study melanoblasts in isolation and also with respect to other neural crest derivatives.

A small proportion of the Tg(Dct;CFP) lines generated also displayed ectopic expression of CFP. In some cases we had seen the same ectopic expression in our initial trials with the mouse *Dct* promoter, for example CNS expression, and so they were expected. We know that *Dct* is expressed in mouse telecephalon so it is conceivable that the mouse *Dct* promoter, when used in zebrafish, could drive reporter gene expression in the CNS. Other patterns were unexpected, and might best be explained by the suggestion that the site of integration of the transgene can effect the expression pattern; this might also explain the slight variation in strength of CFP signal between the different lines (Kawakami 2004). Indeed, the Tol2 system has been used to take advantage of insertional effects in a gene trap approach whereby endogenous promoters drive expression of Tol2 inserted GFP (Kawakami et al. 2004). One of our lines which produced an ectopic expression pattern could be very useful; Chris has CFP-labelled

xanthophores. As we noted in Chapter 3, this cell type currently only has two published markers and these have limitations since they also appear to mark early melanoblasts (Parichy et al. 2000b). Of course a more thorough characterisation of the line would be required but it could prove valuable.

Another of the ectopic expression patterns we observed in the Tg(Dct;CFP) lines was seen in the Clive line. F2 embryos from the Clive line occasionally had expression in the heart as well as in melanoblasts/melanophores. That this pattern was only seen in a small number of embryos in a batch from any given pair of fish suggests that there may be multiple insertion sites of our transgene resulting in multiple expression patterns from the same founder. Whilst the Tol2 system ensures that only a single copy of the transgene is inserted at any one site, it may be inserted multiple times at different sites within the genome. More than 25 insertion sites have been transmitted by a single founder but with an average of 5.6 insertions per founder, much lower numbers are more usual (Kawakami 2004). The overall weakness of expression in our transgenics as compared to other lines available in the lab might also be explained by the fact that the Tol2 system ensures single copy insertion. Other lines, such as Tg(-4.9*sox10*GFP) have been generated by injecting linearised plasmid. This method has been shown to lead to concatemerisation of the DNA before integration which leads to increased reporter expression in the embryo (Stuart et al. 1988).

We successfully generated a pTol2DctCFP*sox10* plasmid and we were able to show that the CFP-Sox10 fusion is functional as transient expression of it in *sox10*<sup>3</sup> mutants rescues pigment cells. It is interesting that by expressing *sox10* in cells which are already expressing *dct* we were able to rescue xanthophores and iridophores as well as melanophores. This suggests that cells which are expressing *dct* in *sox10*<sup>3</sup> mutants are not yet committed to a melanophore fate. It may simply be that a lack of endogenous *sox10* in these cells means that they have not developed normally at all and so they are not able to commit to the melanophore lineage. However, it is also suggestive of the concept of a pigment cell precursor not yet fully described in wild type embryos which is expected, for a short time, to express differentiation genes indicative of multiple cell types (Lopes et al. 2008). Perhaps in a *sox10* mutant these cells get caught at this stage of differentiation? This is also reminiscent of our observations of the expression of other pigment marker genes in *sox10*<sup>3</sup> mutants in Chapter 3. The expression pattern of the melanophore marker *silva* for example was so extensive it seemed unlikely that it did not overlap with the xanthophore maker *xdh*. We suggested some experiments in Chapter 3 looking for overlap of melanophore and xanthophore marker gene expression in *sox10*<sup>3</sup> mutants to test this possibility. In testing our CFP-Sox10 fusion we did not observe any rescued non-pigment neural crest derived cells but it would

be interesting to look for rescue of such cells, for example enteric neurons. If rescued neurons were found it might indicate that even once cells begin to express genes indicative of a melanophore they retain flexibility in terms of their final fate beyond the pigment cell lineages.

Having established the functionality of our CFP-Sox10 fusion we went on to begin to generate transgenic Tg(Dct;CFP-sox10) lines. We were able to select a number of founders based initially on their CFP expression 27 hours after being injected with pTol2DctCFPsox10 and later by selecting those that generated CFP positive F1 progeny (which we grew and used to establish each line). However, in the F2 generation we could see no CFP expression either as protein or transcript between 24 hpf and 72 hpf. This was true for progeny from all of the eight different founders we examined and is surprising because reports suggest that generation of transgenics by the Tol2 system can allow transgene expression as far as F5 (Kawakami 2005). Of course, the *CFP-sox10* fusion may be expressed at levels below the detection threshold for in situ hybridisation and we have not tested whether any such expression could be detected by RT-PCR. Nevertheless, the lack of detectable CFP expression, by fluorescence microscopy or by ISH, meant that the *CFP-Sox10* fusion was not expressed in our F2 embryos at levels that would enable us to test the hypothesis that overexpression of Sox10 in melanophores would halt or delay their development. An alternative approach might be to generate transient transgenics with which to test our hypothesis. However, we found that less than 1 % of embryos injected had any CFP-expressing cells. In addition, there were usually only 1-3 CFP-expressing cells within those embryos some of which were not melanoblasts/melanophores. Therefore, for practical reasons it was unfeasible to generate enough transient transgenics with enough CFP positive cells to be able address the hypothesis in this way.

It might be possible to try the same experiment again, perhaps using the Fugu *tyrp1* promoter which was reported in Zou et al. (2006) as being melanophore specific at least until 3 dfp. Of course, if silencing occurs because of the incorporation of a *CFP-sox10* fusion into the genome rather than due to effects of the promoter, then silencing may occur again. Use of a heatshock promoter (Pyati, Webb and Kimelman 2005) or GAL4/UAS system (Halpern et al. 2008) with our *CFP-sox10* fusion would allow control over the timing of transgene expression and may prevent silencing. A complimentary approach might include overexpression of Sox10 in melanophores in culture. Zebrafish melanophore culture is not well documented so care would have to be taken here to carefully control culture conditions and to fully understand how normal melanophore development in vitro proceeds before attempting to disrupt it by overexpression of *sox10*. Alternatively, if Sox10 must be downregulated to allow

melanophore differentiation we would predict that there would be Sox10 binding sites within the promoters of melanophore differentiation genes which would allow Sox10 to repress their expression. This could be tested by utilising in vitro promoter analysis (Antonellis et al. 2006) to determine whether there are Sox10 binding sites within, for example, the zebrafish *tyrosinase* promoter and whether expression from the *tyrosinase* promoter can be directly inhibited by high levels of Sox10.

Another interesting observation from our PCR analysis of F1 and F2 fish is that there are several different products which can be amplified using our primers. Unexpectedly, we found that we were amplifying sequence from the vector backbone as well as the expected CFP sequence. Integration of Tol2 vector has been reported previously but only when the transgene has also been inserted (Kawakami 2004). Here we see examples where only the vector becomes incorporated into the genome (Figure 4.19 B, sample 7). This could represent bias in the PCR to generate only one of two or more possible products. However, we do see all combinations of PCR products in different PCRs carried out under the same conditions. Further to the observation of multiple PCR products, progeny from F1 fish 19 segregate according to whether they have the smaller, larger or both of the two PCR bands produced when fish 19 is genotyped (Figure 4.19 D). This suggests that the transgene and vector sequence have been inserted separately at different integration sites in one founder fish. Multiple insertion sites have been reported previously (Kawakami 2004) but again, not in this combination of transgene and vector sequences. Whether or not it matters that vector sequence can be integrated in this way requires investigation. In our study, it could be that this has disrupted our transgene in some way or somehow triggered silencing of the promoter. It might be interesting to explore these possibilities by finding out, within individual fish, which sequences are incorporated from our construct, how many times and in what combinations. Incorporation of vector sequence independent of transgene sequence could cause a problem when making any transgenic but particularly in those used in insertional mutagenesis screens. Depending upon the insertion site, vector sequence could disrupt expression of endogenous transcripts and cause a phenotype whilst remaining undetected. That phenotype may then be incorrectly attributed to the disruption at the site of the intended transgene insertion. This may not occur often, and would usually be seen as segregation of phenotypes, but it may be an important possibility to consider when using transgenics in this way.

### 4.3.3 Summary

We have shown in this Chapter that Sox10 protein and mRNA are downregulated in melanophores as they develop. We were also able to successfully generate two kinds of transgenic lines to test the prediction that Sox10 must be downregulated to allow



melanophores to differentiate. One type of transgenic expresses CFP under the control of the mouse *Dct* promoter, in many cases faithfully recapitulating the endogenous *dct* expression pattern. We also generated other lines which incorporate the same promoter along with *CFP* sequence fused to *sox10*. However, we were unable to detect any expression of CFP in the F2 generation of any of our Tg(Dct;CFP-sox10) lines and it is unlikely that any of our lines were overexpressing Sox10 as we had intended. Certainly no obvious melanophore phenotype was visible. As such, our prediction that Sox10 must be downregulated to allow melanophores to differentiate remains open for testing.

# Chapter 5

## Living Test Tubes

### 5.1 Introduction

As we discussed in Chapter 1, there is a lot of information from different organisms about the different genes that Sox10 may be able to regulate in different cell types. Our model for melanophores predicts only indirect regulation of melanophore marker genes by Sox10 because here Sox10 works via activation of *mitfa* which then directly regulates melanophore differentiation genes (Figure 1.04). In zebrafish, this transient role for Sox10 is supported by data showing that overexpression of *mitfa* in the neural crest of either Sox10 or Mitfa deficient embryos can rescue melanophores to the same degree in each (Elworthy et al. 2003). However, in mouse it seems that Sox10 has an additional role beyond activation of *Mitf*, to assist in the activation of *Tyrosinase* expression (Hou, Arnheiter and Pavan 2006). This leads to the conclusion that in mice *Mitf* alone is not sufficient for melanocyte differentiation. This conclusion is contrary to work from other groups which have shown the overexpression of *Mitf* in mouse fibroblasts, quail neuroretina cells and in both zebrafish and medaka, to be sufficient for expression of all of the *Tyrosinase* family genes and for pigmentation (Tachibana et al. 1996, Planque et al. 1999, Béjar, Hong and Schartl 2003, Lister et al. 1999). There are other contradictory data about the possibility of later roles for Sox10 in melanocyte development. In cell culture, Sox10 can activate expression from the *Dct* promoter (Britsch et al. 2001, Potterf et al. 2001, Jiao et al. 2004) suggesting Sox10 may play a later role in regulating *Dct*. However, Sox10 is not necessary for expression of *Dct* in neural tube culture if *Mitf* is present (Hou et al. 2006) and *dct* can be induced by ectopic expression of only *mitfa* in zebrafish (Lister et al. 1999) which suggests that a late role for Sox10 may not exist or at least is not vital for *Dct* expression. Hou et al. (2006) conclude that their work demonstrates contrasting roles for Sox10 in melanocyte development in zebrafish and mouse. They explain the disparity between current findings as representing actual differences between the two

species. We feel that due to the contradictions in the literature outlined above, further experimentation is required before this proposal can be confirmed. Ideally, the mouse data needs to be tested *in vivo* to validate *in vitro* findings. A study in zebrafish to explore exactly which genes are regulated by Sox10 and Mitfa in this model organism would be complimentary to current data in zebrafish. It would also aid comparison with data from the available mouse studies since it would provide a closer parallel to these studies.

Previously, injection of *sox10* RNA into one cell stage zebrafish embryos has been shown to induce expression of *mitfa* by 6 hpf as assayed by ISH (Elworthy et al. 2003). Similar injection of *mitfa* RNA will induce expression of *dct* (Lister et al. 1999). We will utilise this method, which treats the zebrafish embryo almost as a ‘living test tube’, to look at which of our melanophore marker genes (*dct*, *tyrosinase*, *tyrp1b* and *silva*) can be induced by Sox10 or Mitfa, both alone and in combination. From our original model, we would predict that overexpression of *mitfa* in this way will lead to expression of all of our marker genes and that overexpression of *sox10* will not directly induce expression of our marker genes. If *sox10* is overexpressed in addition to *mitfa* however, the model predicts that it will repress any expression of melanophore marker genes induced by *mitfa*. On the other hand, if data from Hou et al. (2006) can also be applied to zebrafish we will see expression of *dct*, *silva* and *tyrp1b* after overexpression of *mitfa* but we will not see expression of *tyrosinase* unless we additionally overexpress *sox10*. Thus, we have a set of clear predictions to test in this chapter.

## 5.2 Results

We generated sense mRNA for *sox10*, *sox10*<sup>L142Q</sup> (which we will refer to as *L142Q*), *mitfa* and *mitfa*<sup>w2</sup> (which we will refer to as *w2*). *Sox10*, *L142Q* and *w2* RNA were diluted to a concentration of 25 ng/μl. We found this dose of *mitfa* RNA to be lethal so it was diluted to a concentration of 6.25 ng/μl. We injected embryos with 4.6 nl RNA and grew them for six or 10.5 hours (i.e. prior to the onset of endogenous expression of *sox10* or *mitfa*). Embryos were then processed for *in situ* hybridisation to see whether expression of our genes of interest had been induced. All of the experiments in this section were repeated on at least two separate occasions. Whenever we injected wild type *sox10* or *mitfa* RNA we also injected a set of embryos with *L142Q* or *w2* mutant RNA respectively as negative controls.

### 5.2.1 Sox10 Indirectly Induces Expression of *tyrp1b*

We first injected *sox10* RNA into wild type embryos and looked for expression of *mitfa*. As expected (Elworthy et al. 2003), six hours after injection we saw expression

of *mitfa* in 55 % of embryos (Table 5.01). This validated our method and technique. In the same experiment we also looked for expression of *dct*, *silva*, *tyrosinase* and *tyrp1b*. We did not see expression of any of these marker genes in either our experimental embryos or our negative controls at 6 hpf (Figure 5.01 A). We next addressed whether allowing embryos to grow to 10.5 hpf altered the result (Figure 5.01, B). Now, we found that although a similar proportion of embryos were positive for *mitfa* (50 %) and none were positive for *dct*, *silva* or *tyrosinase*, we did see expression of *tyrp1b* in 46 % of embryos (Table 5.01). Again, there was no expression of any of these genes after injection with *L142Q* RNA. We know that *mitfa* is directly activated by Sox10 and since *tyrp1b* expression did not appear until four and a half hours later, it seemed likely that this was as a result of indirect activation by Sox10 via Mitfa. To test this, we injected *sox10* RNA into embryos from an *mitfa*<sup>w2</sup> homozygous mutant incross and looked for expression of *tyrp1b* after 10.5 hours. We saw no *tyrp1b* expression in 160 embryos. This supports the hypothesis that the *tyrp1b* expression we see in wild type embryos injected with *sox10* RNA is *mitfa* dependent and strongly supports the hypothesis that *tyrp1b* is not directly regulated by Sox10 under these conditions.

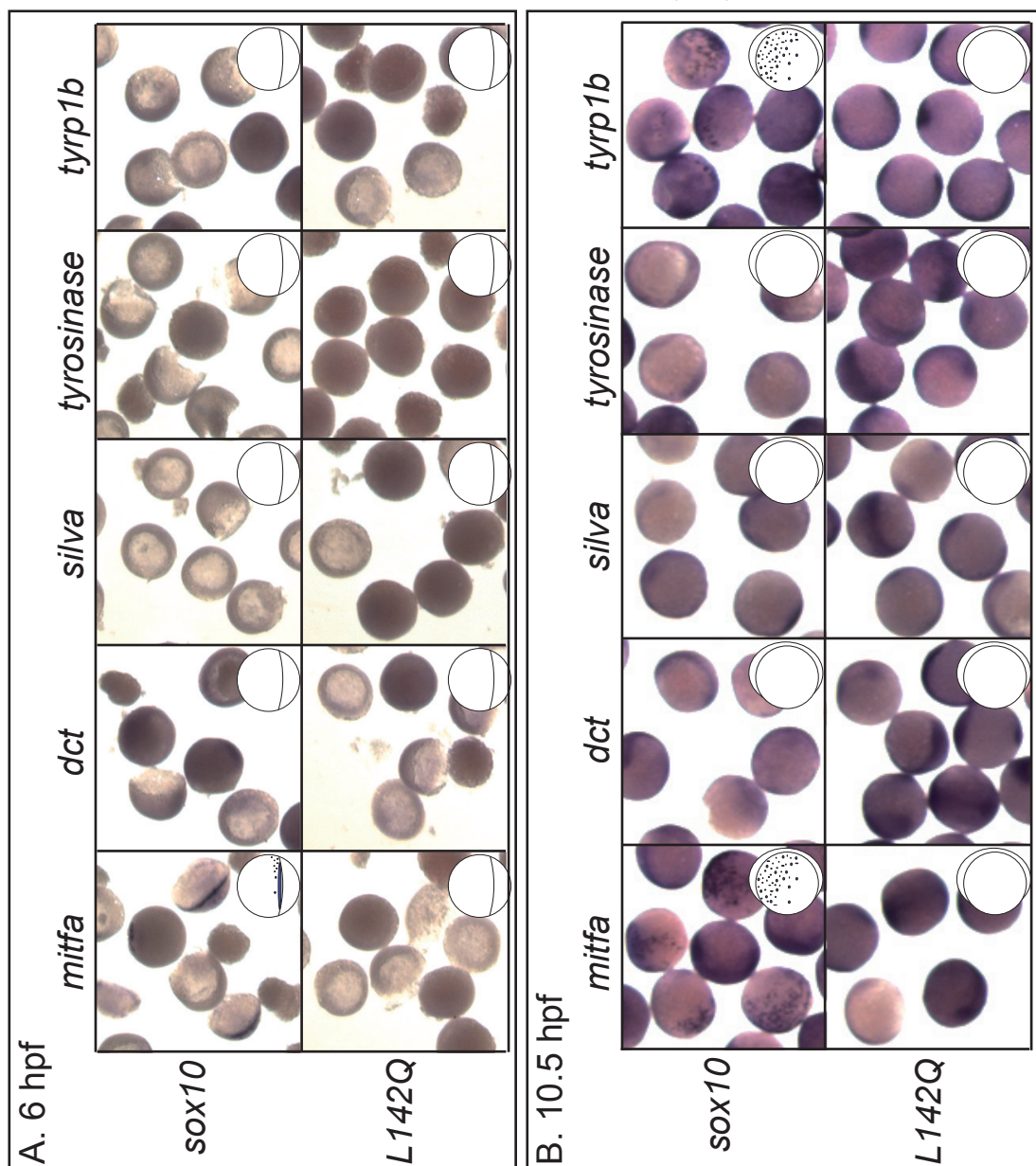
### 5.2.2 Mitfa Can Induce Expression of *dct*, *silva*, *tyrosinase*, *tyrp1b* and *sox10*

We would have predicted that if expression of *tyrp1b* is *mitfa* dependent, then *mitfa* should also be able to activate expression of the other melanophore marker genes. Indeed, based on our model we would predict that injection of *mitfa* RNA will induce expression of our marker genes. However, from Hou et al. (2006) we would predict that this will not be sufficient for expression of *tyrosinase*. We now tried injecting *mitfa* RNA into wild type embryos and asked which genes might be turned on by this high level of *mitfa* RNA in the absence of *sox10*. We found that all of our melanophore marker genes were expressed in 6 hpf embryos after injection with *mitfa* RNA (Figure 5.02). The pattern and quantity of expressing cells in each embryo was highly variable but expression was seen in most embryos injected with wild type RNA (Table 5.02) but not at all in embryos injected with control *w2* RNA. We also tested some of the same embryos to confirm that there was no expression of *sox10*. Unexpectedly however, we did see expression of *sox10* induced by injection of *mitfa* RNA in 89 % of embryos. This was completely unexpected since regulation of *sox10* by Mitfa has never been reported previously, although a lack of regulation has not been reported either. The result is also in direct contradiction to the prediction from our model that Mitfa will have a repressive effect upon *sox10* expression.

This unexpected result also raised the issue of whether the expression of our melanophore markers after injection with *mitfa* RNA was dependent upon the induced expression

Table 5.01 Expression of marker genes after injection with *sox10* RNA

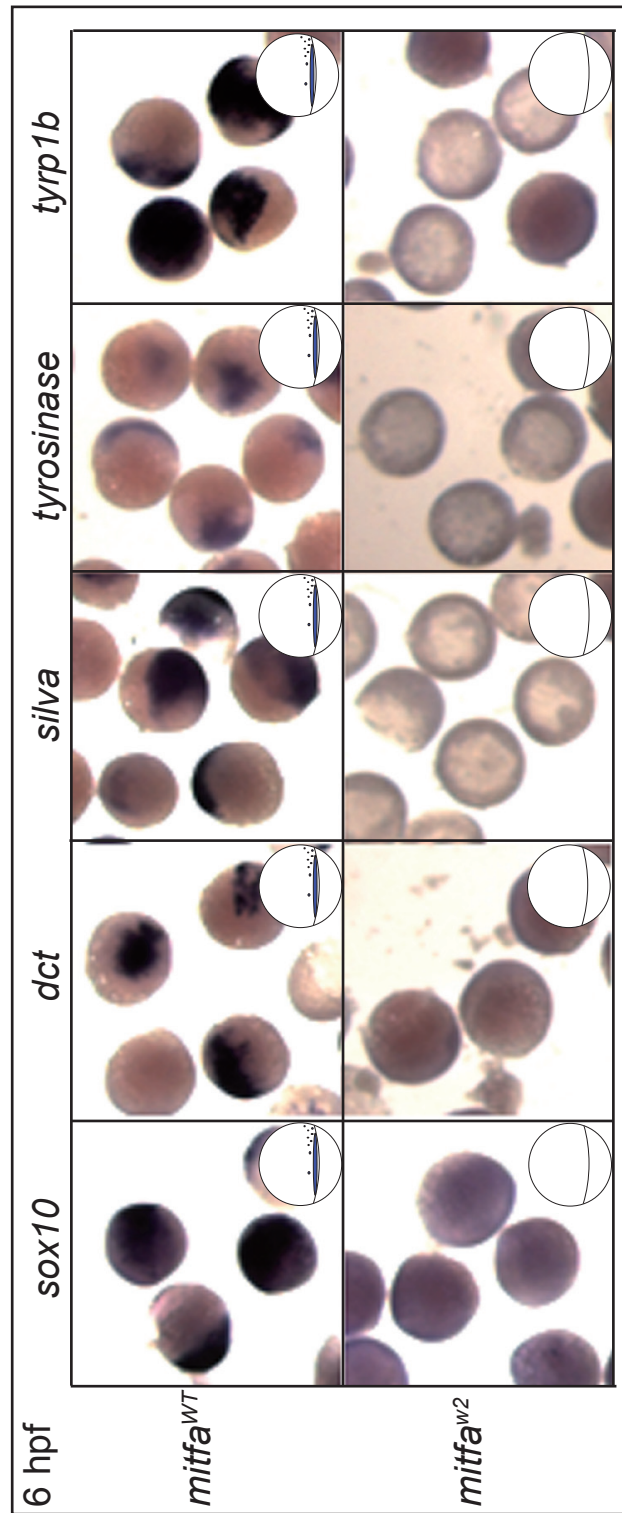
	<i>mitfa</i>	<i>dct</i>	<i>silva</i>	<i>tyrosinase</i>	<i>tyrp1b</i>
<b>6 hpf</b>					
<i>sox10</i>	34/62	0/64	0/63	0/63	0/63
	55 %	0 %	0 %	0 %	0 %
<i>L142Q</i>	0/58	0/61	0/61	0/61	0/62
	0 %	0 %	0 %	0 %	0 %
<b>10.5 hpf</b>					
<i>sox10</i>	40/51	0/81	0/71	0/82	39/84
	78 %	0 %	0 %	0 %	0 %
<i>L142Q</i>	0/71	0/73	0/73	0/73	0/70
	0 %	0 %	0 %	0 %	0 %



**Figure 5.01 Induction of melanophore markers by *sox10* RNA**

A and B. Figures illustrating the embryos after *sox10/L142Q* RNA injection and ISH processing. Also shown are schematics to aid interpretation. Note, embryos injected with *L142Q* are always negative for marker gene expression. A. *sox10* RNA injected into wild type embryos induces only expression of *mitfa* by 6 hpf. B. *sox10* RNA injected into wild type embryos will additionally induce expression of *tyrp1b* by 10.5 hpf. Expression of *dct*, *silva* and *tyrosinase* are never induced in this way.





**Figure 5.02 Expression induced by injection of *mitfa* RNA**

Expression of *sox10*, *dct*, *silva*, *tyrosinase* and *tyrp1b* are all induced six hours after injection of *mitfa* RNA into wild type embryos. Injection of a mutant *mitfa* RNA, *w2*, causes no such induction. For future reference, note also the schematics.

Table 5.02 *mitfa* injection into wild type embryos

RNA	<i>sox10</i>	<i>dct</i>	<i>silva</i>	<i>tyrosinase</i>	<i>tyrp1b</i>
<i>mitfa</i>	55/62	46/54	40/45	39/46	39/44
	89 %	85 %	89 %	85%	89 %
<i>w2</i>	0/44	0/63	0/64	0/61	0/63
	0 %	0 %	0 %	0 %	0 %

Between 85 % and 89 % of embryos were positive for each of our markers. No expression was ever seen in the control embryos.

of *sox10*. To test this we repeated the experiment above on embryos from crosses between *sox10*<sup>l3</sup> heterozygous fish. These experiments were repeated on at least three occasions. 100 embryos from each set of experiments were left uninjected and scored at 3 dpf to ensure that we had 25 % mutant embryos. We would predict that if Sox10 is not required for the expression of any of our marker genes then all of the embryos will express *dct*, *silva*, *tyrosinase* and *tyrp1b* after *mitfa* injection. If Sox10 is required for any one gene to be expressed, then the embryos which are *sox10*<sup>l3</sup> mutants (25 %) will not express that gene. The analysis of the results of this experiment is complicated by the fact that even in wild type embryos, after injection with *mitfa* RNA, only 85 % - 89 % express the markers. So in fact, if Sox10 is necessary for expression of our marker genes we expect the proportion of embryos not expressing each gene to be *at least* 25 % of the total number of injected embryos.

Embryos from a *sox10*<sup>l3</sup> heterozygous incross injected with *mitfa* RNA expressed *dct*, *silva* and *tyrp1b* in 86 %, 91 % and 94 % of embryos respectively. The proportion of embryos which did not express each gene differed significantly from the minimum 25 % that we would expect if Sox10 is necessary for their expression (Two tailed Chi squared test, df = 1, p<0.001). We conclude that the expression of *dct*, *silva* and *tyrp1b* are independent of *sox10* but dependent upon *mitfa* expression.

In the same experiment, we saw *tyrosinase* expression in 73 % of embryos. This does not differ significantly from 75 % (Two tailed Chi squared test, df=1, p>0.05) and suggests that *sox10* may be required, together with *mitfa*, for *tyrosinase* expression. However, even in wild types there is not complete penetrance (only 85 % of wild type embryos showed *tyrosinase* induction) so we would actually expect much less than 73 % of embryos to express *tyrosinase* if its expression is dependent upon both Sox10 and Mitfa. Whilst we cannot rule out that *sox10* is required for *tyrosinase* expression we feel that the data presented here suggests it is not.

### 5.2.3 Mitfa Can Induce Expression of GFP from 7.2 kb of the *sox10* Promoter

That Mitfa can induce expression of *sox10* is surprising and is contradictory to our model which suggests that Mitfa will actually repress expression of *sox10*. We utilised a transgenic line available in the lab, Tg(-7.2*sox10*:GFP), which expresses GFP under the control of 7.2 kb of the zebrafish *sox10* promoter to ask whether Mitfa can induce expression from that region of promoter. We injected *mitfa* RNA into one cell stage embryos from an outcross of heterozygous transgenic fish. If, as we predict, Mitfa is able to bind within the 7.2 kb of *sox10* promoter present in the line, it will activate expression of GFP. This is what we saw in 94 out of 257 embryos (37 %), six hours after

injection (Figure 5.03 and Table 5.03). At 10.5 hpf the proportion of GFP expressing embryos had increased slightly to 45 %. Both of these figures are lower than the 86 % of embryos which express *sox10* RNA after *mitfa* injection. However, since only 50 % of embryos will be transgenic and if the effect of activation of the endogenous and the transgenic promoters are equivalent we only expect approximately 45 % of embryos to be GFP positive. No GFP expression was seen in any control embryos injected with *w2* RNA. We have shown that *mitfa* can activate expression of *sox10* and that 7.2 kb upstream of the *sox10* ATG is sufficient for this to occur thus localising likely Mitfa response elements.

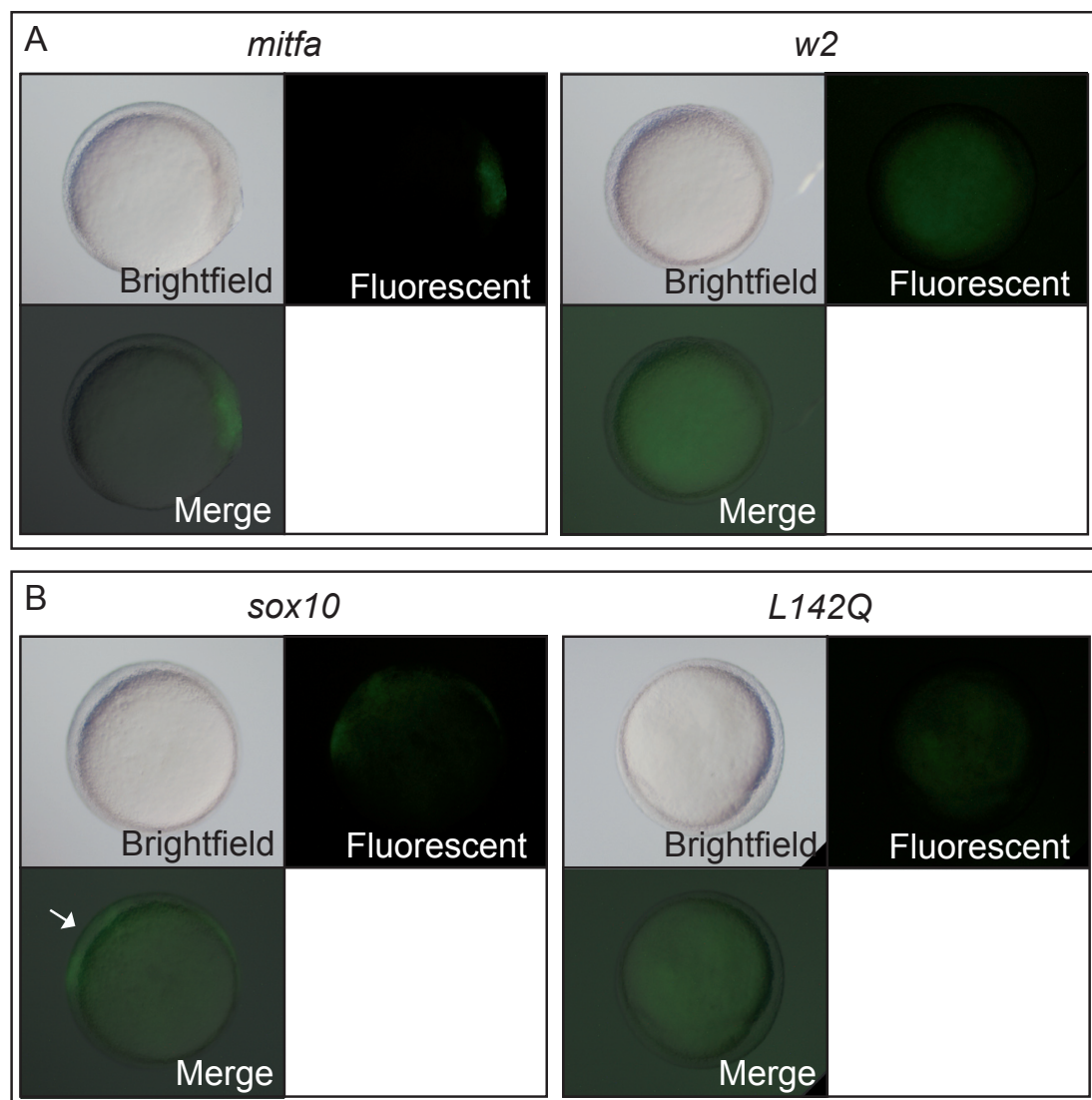
We also injected *sox10* RNA into Tg(-7.2*sox10*:GFP) transgenic embryos. Six hours after injection we saw only 6 % of embryos had GFP expression but that by 10.5 hours after injection, 49 % of embryos had GFP expression (Figure 5.03 and Table 5.03). Again, injection of *L142Q* RNA had no effect.

We analysed the 7.2 kb *sox10* promoter for existence of potential Mitf binding sites. This revealed six potential Mitf binding sites at -997 bp, -1143 bp, -3482 bp, -4992 bp, -5152 bp and -6703 bp upstream of the translation start site (Figure 5.04). The latter three of these would be absent from the promoter sequence used in the Tg(-4.9*sox10*:GFP) line.

### 5.2.4 Sox10 Inhibits Activation of Marker Genes by Mitfa

Our original model (modified based on earlier results) predicts that *sox10* will have an inhibitory effect upon the activation of our marker genes (except *tyrp1b*) by *mitfa* but we hypothesised that this would only occur if the timing of *sox10* and *mitfa* expression is appropriate. Thus, when *mitfa* RNA is injected into embryos we see expression of all of our marker genes at 6 hpf. This may be because the delayed expression of *sox10* that we also see in these embryos is not present early enough or in sufficient quantities to inhibit the action of *mitfa*. When *sox10* RNA is injected into embryos it is present well before *mitfa* and so can inhibit expression of our marker genes by *mitfa* so that we only see expression of *tyrp1b*. *tyrp1b* expression does not appear until 10.5 hpf because it is *mitfa* dependent and requires time for Mitfa to build up. These ideas are also presented in Figure 5.05.

To test this hypothesis, we injected embryos with a mixture of *sox10* and *mitfa* RNA. We predicted that the presence of high levels of *sox10* from the start of the experiment would inhibit the expression of our marker genes that we would have seen if only *mitfa* RNA was injected. We used the same concentration of each RNA as had previously been used. This meant that the total RNA injected was higher but this did not appear



**Figure 5.03 Injection of *mitfa* and *sox10* RNA into Tg(-7.2*sox10*:GFP) embryos**

A. Brightfield, fluorescent and merged images of 6 hpf embryos. Left, these embryos had been injected with *mitfa* RNA. GFP expression can clearly be seen on the righthand side of the embryo. Right, these embryos had been injected with *w2* RNA. Apart from autofluorescence from the yolk, no GFP expression is seen.

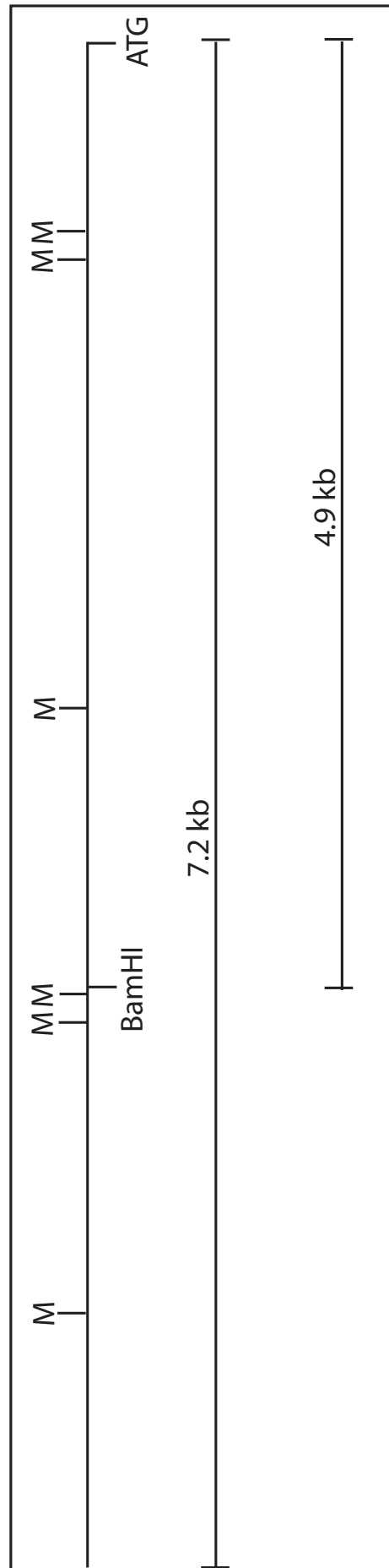
B. Brightfield, fluorescent and merged images of 6 hpf embryos. Left, these embryos had been injected with *sox10* RNA. GFP expression can clearly be seen in the top of the embryo (arrow). Right, these embryos had been injected with *L142Q* RNA. Apart from autofluorescence from the yolk, no GFP expression is seen.

Table 5.03 Expression of GFP after *mitfa* or *sox10* RNA injection after 6 or 10.5 hours

RNA	6 hpf		10.5 hpf	
<i>mitfa</i>	94/257	37 %	68/152	45 %
<i>w2</i>	0/118	0 %	0/78	0 %
<i>sox10</i>	20/319	6 %	95/193	49 %
<i>L142Q</i>	0/120	0 %	0/80	0 %

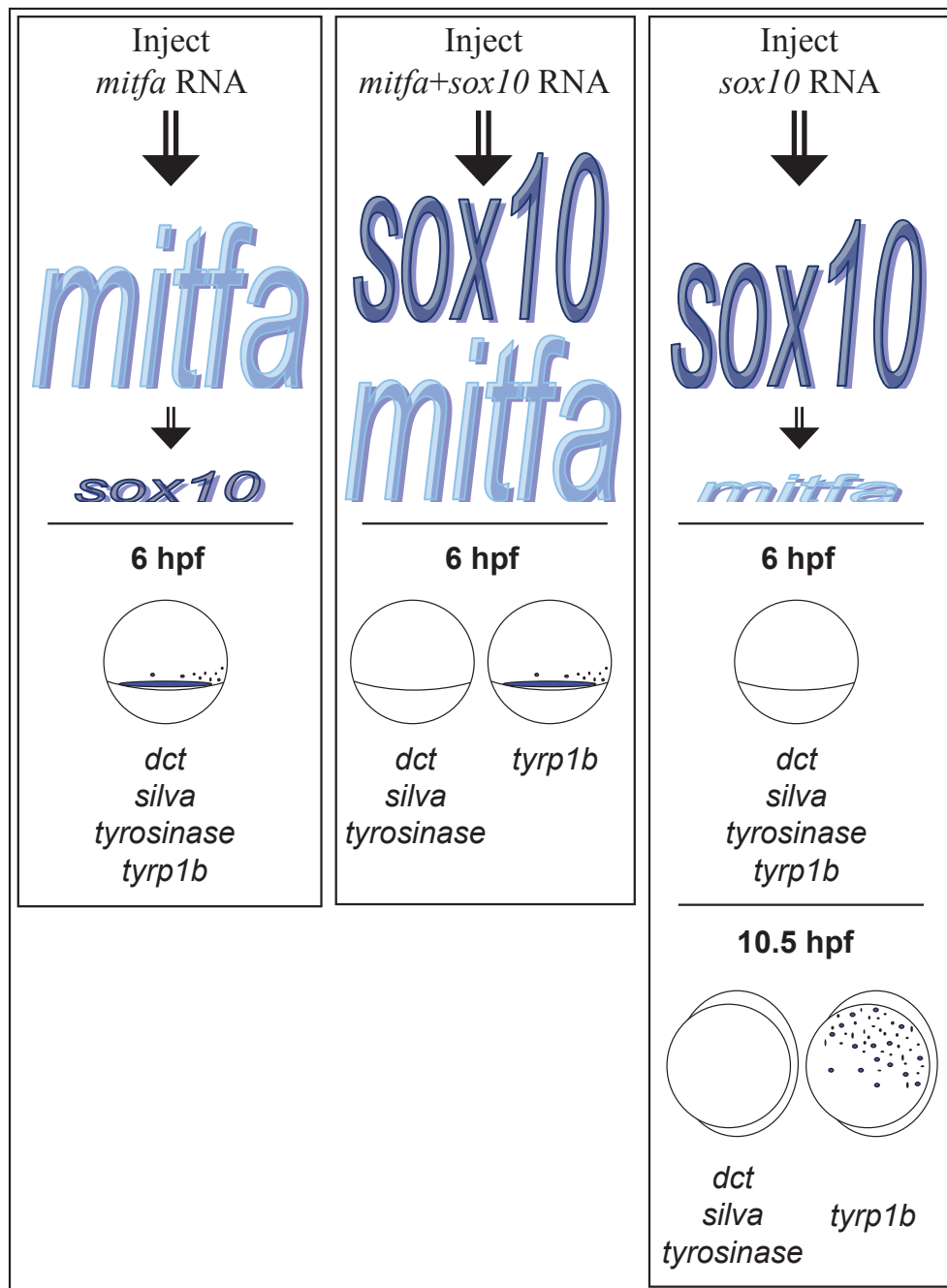
Numbers are reduced compared to *sox10* as measured by ISH (Table 5.01) since only half of these embryos are transgenic. Note the increase in GFP positive embryos between 6 hpf and 10.5 hpf after injection with *sox10* RNA.





**Figure 5.04 Mitf binding sites in the 7.2 kb *sox10* promoter**

There are six predicted Mitf binding sites (M) in the 7.2 kb *sox10* promoter at positions -997, -1143, -3482, -4992, -5152, -6703 relative to the translation start site. Three of these are absent from the first 4.9 kb of the promoter, delineated by a BamHI site.



**Figure 5.05 Timing of *sox10* and *mitfa* expression are important**

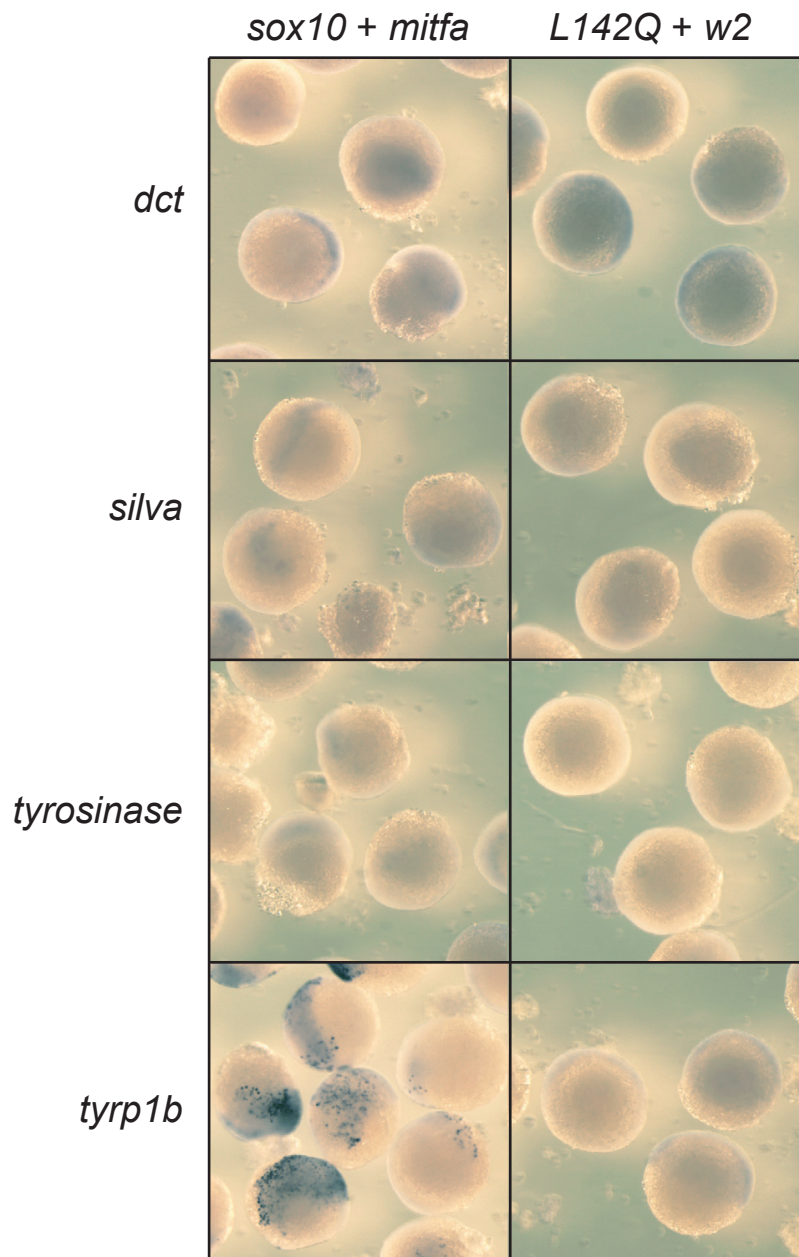
Injection of *mitfa* RNA induces expression of *sox10* as well as all of our other markers at 6 hpf (first column). If we inject *sox10* RNA, we only see expression of *mitfa* at 6 hpf but by 10.5 hpf we also see expression of *tyrp1b* (third column). We hypothesised that *sox10* is able to repress expression of *dct*, *silva* and *tyrosinase* but only when it is present in the embryo early enough and at high enough levels compared to *mitfa*. This would explain why we see different expression profiles seen when we injected *mitfa* or *sox10* RNA alone despite the eventual expression of both genes in both experiments. We predicted that injection of *mitfa* and *sox10* RNA together, so that both RNAs are present from the beginning of the experiment and at high levels, will result in the same profile of gene expression at 6 hpf as if we injected *mitfa*, except that due to the high levels of *sox10* present from an early stage, *dct*, *silva* and *tyrosinase* will not be expressed (second column).

to affect the embryos. We found that six hours after injection, *tyrp1b* was expressed but none of the other markers were (Figure 5.06 and Table 5.04). This is the same result as if we had only injected *sox10* RNA, except that we see *mitfa* dependent expression of *tyrp1b* 4.5 hours earlier due to the presence of *mitfa* RNA from the beginning of the experiment on this occasion. This result supports the hypothesis that *sox10* can have an inhibitory effect upon *dct*, *silva* and *tyrosinase* expression but not *tyrp1b* expression.

### 5.2.5 Levels of Sox10 Alter the Functions it Performs

To explore further the inhibitory role of Sox10, we carried out another experiment. We wanted to know whether, when mixed with *mitfa* RNA, lower concentrations of *sox10* RNA than we used previously would allow some expression of *dct*, *silva* or *tyrosinase*. Kim et al. (2003) were able to show that low levels of Sox10 are sufficient to induce expression of Mash1 and Phox2b in sympathetic neurons but much higher levels are required to inhibit neuron differentiation. Perhaps in our model system Sox10 might also be required at high levels in order to inhibit expression of our melanophore marker genes. We made five different mixtures of *sox10* and *mitfa* RNA which we injected into wild type embryos. The first was the same as we used in previous experiments, 25 ng/μl *sox10* plus 6.25 ng/μl *mitfa*. The four remaining mixtures contained 6.25 ng/μl *mitfa* plus either 12.5 ng/μl, 6.25 ng/μl or 3.125 ng/μl or 0 ng/μl of *sox10* RNA with the remainder of the total amount of RNA being made up with *L142Q* (Figure 5.07, A). For any one RNA mixture, all of the embryos required for the experiment were injected in succession and grown together. Embryos were only divided into separate tubes to be processed for ISH with different markers immediately prior to beginning the ISH protocol. The experiment was repeated three times. On the first two occasions each mixture was made up individually. On the third occasion, a serial dilution of the *sox10* RNA was performed in an attempt to increase accuracy of the dilutions.

The data we obtained from these experiments was noisy but the general trend observed was that as the concentration of *sox10* RNA that was injected decreased, a higher percentage of embryos expressed each marker gene (Figure 5.07). For *dct* as well as *tyrosinase* (Figure 5.07, B and D) both of the end points are consistent with previous experiments (most embryos express the markers when *L142Q* is injected at 25 ng/μl with *mitfa* RNA at 6.25 ng/μl and none or very few do when *sox10* is injected at 25 ng/μl with *mitfa* RNA at 6.25 ng/μl). The averaged data for *dct* shows a smooth curve with no points which seem anomalous (light blue) and the experimental data follows this trend, although there are anomalous data points. The averaged *tyrosinase* curve appears to begin more steeply and flatten sooner than that for *dct* and all three experimental curves fit reasonably closely to the averaged data curve with only one

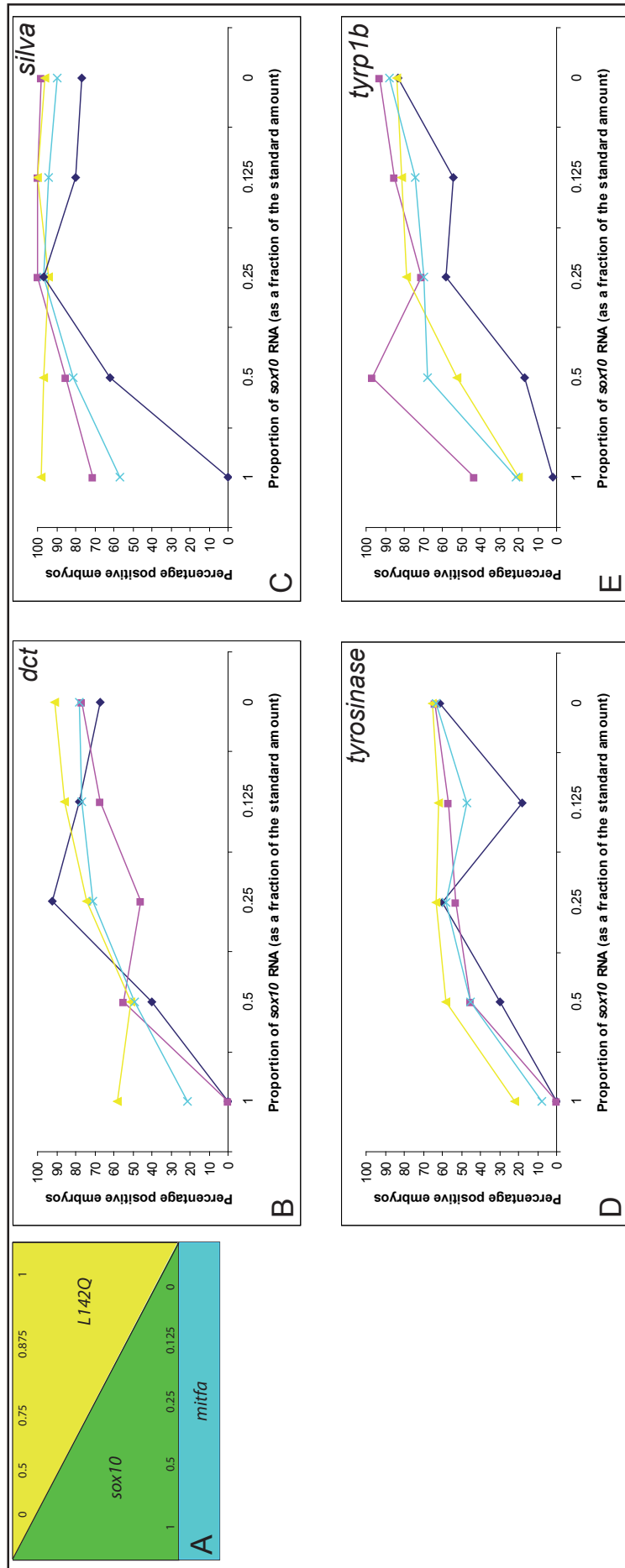


**Figure 5.06 Marker gene expression 6 hours after injection of *sox10* and *mitfa* RNA**

As predicted, only *tyrp1b* is expressed when embryos are injected with a mixture of *sox10* and *mitfa* RNA. Negative controls, injected with *L142Q* and *w2* RNA, are all negative.

Table 5.04 Expression of marker genes after injection with *sox10* and *mitfa* RNA together

	<i>dct</i>	<i>silva</i>	<i>tyrosinase</i>	<i>tyrp1b</i>
<i>sox10</i> + <i>mitfa</i>	0/85	0/116	0/119	65/100
	0 %	0 %	0 %	65 %
<i>L142Q</i> + <i>w2</i>	0/52	0/73	0/79	0/67
	0 %	0 %	0 %	0 %



**Figure 5.07 Injection of *mitfa* RNA with different amounts of *sox10* RNA, alters marker gene expression**

A. Diagram to illustrate the mixtures of RNA used. *mitfa* (blue) concentration remains constant whilst *sox10* (green) decreases from the standard (1) and *L142Q* (yellow) increases.

B-E Graphs representing changes in the numbers of embryos which express each of our marker genes in the presence of different levels of *sox10* RNA. Embryos were injected with *mitfa* RNA mixed with *sox10* RNA. The first and last data points mimic data previously gathered (standard *sox10* concentration, 1 and no *sox10* RNA, 0). In between *sox10* concentration was, half, one quarter or one eighth of the standard. Total RNA concentration was balanced with *L142Q* RNA. Dark blue and pink lines represent two experiments where each dilution was measured out separately. The yellow lines represent an experiment where a serial dilution of *sox10* RNA was performed. The light blue lines represent a mean of all three experiments.



anomalous point. There does not seem to be any consistency between markers as to where anomalous points occur that makes them easy to explain.

The results for *silva* are only consistent with previous data where the RNA mixture contained no *sox10* RNA. Except for on one occasion, we see greater numbers of embryos expressing *silva* at the maximum *sox10* dose than we had previously seen and there is little change in the numbers of embryos expressing *silva* as we decrease the concentration of *sox10* (Figure 5.07, C). The results for *tyrp1b* also differ from what we would have expected. In contrast to the other markers, we would expect to see between 50 % and 75 % of embryos expressing *tyrp1b* when the *sox10* RNA concentration is highest and little change as we decrease *sox10* concentration. Instead we see a curve very similar to that for the other markers, although there does appear to be more variation at each point.

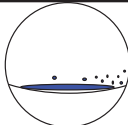
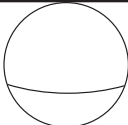
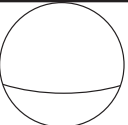
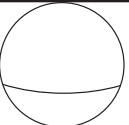
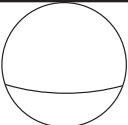
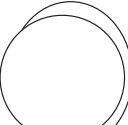
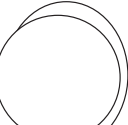
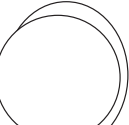
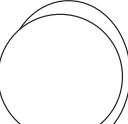
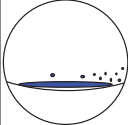
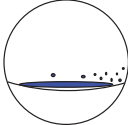
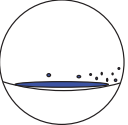
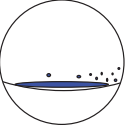
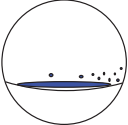
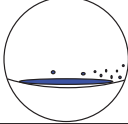
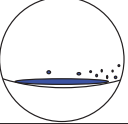
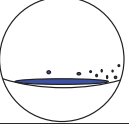
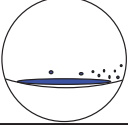
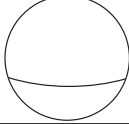
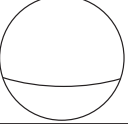
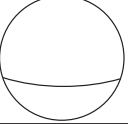
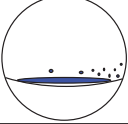
These data do not contradict the hypothesis that *sox10* is only able to inhibit expression of our marker genes when present at high enough levels since we see increased expression of our marker genes as *sox10* concentration decreases. However, it is difficult to make more specific comments about similarities and differences between the genes due to the noise in these experiments.

### 5.3 Discussion

We have tested a model, adapted from one proposed by Kim et al. (2003) for the role of Sox10 in mouse sympathetic neurons, to explain the role of Sox10 in melanophores (Figure 1.04). Our model predicted that whilst Sox10 is required to initiate expression of *mitfa* and begin the process of differentiation, it is also able to repress the expression of differentiation genes downstream of Mitfa. This model also predicts that Sox10 must be downregulated to allow differentiation to proceed and that this downregulation will occur as part of a feedback loop involving Mitfa. Some work in mouse melanocytes contradicts this model and suggests that Sox10 is not only required to initiate expression of Mitf but that it also has a later role, to assist in the induction of *Tyrosinase* expression (Hou et al. 2006). To explore in more detail our original model and enable us to make more direct comparisons between zebrafish and the work presented in Hou, Arnheiter and Pavan (2006), we undertook a set of RNA injection experiments designed to investigate which genes Sox10 and Mitfa may be able to regulate in melanophores. In this chapter we have documented our results from these experiments (summarised in Table 5.05).

With respect to all of our results, it is important to consider that they were obtained

Table 5.05 Summary of data from RNA injections.

Genotype	RNA	<i>sox10</i>	<i>mitfa</i>	<i>dct</i>	<i>silva</i>	<i>tyrosinase</i>	<i>tyrp1b</i>
WT	<i>sox10</i>						
<i>mitfa</i> <sup>w2</sup>	<i>sox10</i>		Not functional				
WT	<i>mitfa</i>						
<i>sox10</i> <sup>3</sup>	<i>mitfa</i>	Not functional					
WT	<i>mitfa</i> + <i>sox10</i>						

*sox10* RNA injected into wild type embryos can induce expression of *mitfa* at 6 hpf and *mitfa* and *tyrp1b* by 10.5 hpf. This *tyrp1b* expression is *mitfa* dependent since we do not see expression of *tyrp1b* after *sox10* injection into *mitfa*<sup>w2</sup> mutant embryos.

*mitfa* RNA injected into wild type embryos can induce expression of all of our marker genes, including *sox10*. However, the expression of these genes is not dependent upon *sox10* expression since we see no significant effect upon marker gene expression when *mitfa* is injected into *sox10*<sup>3</sup> mutant embryos.

When *mitfa* and *sox10* RNA are injected into wild type embryos together we only see expression of *tyrp1b*. This supports the hypothesis that *sox10*, if present early enough and at high levels, represses expression of *dct*, *silva* and *tyrosinase*.

by working in early embryos and the complement of transcription factors here will deviate from that in the developing neural crest. This means that binding partners necessary for normal Sox10 and Mitfa function in the neural crest may not be present, altering the way these proteins function. Equally, there may be additional factors present which are absent from the neural crest and which might also alter the functions of Sox10 and Mitfa. We also worked with fairly arbitrary concentrations of RNA which are not necessarily physiologically relevant so care will need to be taken to assess the physiological relevance of the results obtained.

### 5.3.1 Mitfa Induces Melanophore Marker Gene Expression

Our model clearly predicts that expression of Mitfa is sufficient to induce expression of melanophore marker genes. We found that in wild type embryos that had been injected with *mitfa* RNA and allowed to grow to 6 hpf *sox10*, *dct*, *silva*, *tyrosinase* and *tyrp1b* are all expressed. By repeating the experiment in *sox10<sup>3</sup>* mutants, we went on to show that the expression of *dct*, *silva*, and *tyrp1b* are not Sox10 dependent and that *tyrosinase* expression is unlikely to be Sox10 dependent. This result supports the hypothesis that *sox10* is not required for the expression of *dct*, *silva*, *tyrosinase* and *tyrp1b*, consistent with the idea that Sox10 is only required to induce expression of *mitfa* after which it is not needed for melanophore development (Elworthy et al. 2003). It also supports work in other organisms which shows that Mitfa is able to induce expression of *Dct*, *Tyrosinase* and *Tyrp1* (Murisier and Beermann 2006, Hou et al. 2006, Baxter and Pavan 2003, Goding 2000, Lowings, Yavuzer and Goding 1992). The lack of a late role for Sox10 in melanophore development suggested by this work is in contrast to mouse data which suggests that Sox10 is required, in addition to Mitfa, for *tyrosinase* to be expressed and for melanophore differentiation to proceed (Hou et al. 2006). It adds weight to the proposal put forward by Hou et al. (2006) that zebrafish and mouse appear to be inherently different in the roles that Sox10 performs in melanocytes. Also in agreement with this paper, but contrary to others which demonstrate a positive role for Sox10 in *Dct* expression in mouse (Britsch et al. 2001, Potterf et al. 2001, Jiao et al. 2004), we have found no evidence to suggest a role for Sox10 in induction of *dct* in vivo in zebrafish. Further work in vivo in mouse would provide interesting evidence to help resolve these contradictions.

Ideally we would have confirmed our results by genotyping the *sox10<sup>3</sup>* embryos and scoring the mutants for whether or not they expressed our markers. We were unable to optimise our PCR genotyping procedure to carry this out. The major hurdles were the small quantities of DNA (from 6 hpf embryos) combined with the fact that the embryos had been through an ISH protocol. The *sox10<sup>3</sup>* genotyping PCR is also difficult since it requires amplification of both a small (423 bp, wild type) band and a large (1.8 kb,

mutant) band. In addition, the difference in size between these two bands is due to a transposon insertion which has repetitive elements for much of its length precluding the design of primers within that region (Carney 2003). Another way to test the result would be to conduct double ISHs on *mitfa* RNA injected embryos to look for co-expression of *sox10* and *tyrosinase* or, as predicted from our original model, *tyrosinase* positive embryos which are not *sox10* positive.

### 5.3.2 Mitfa Can Activate Expression from the *sox10* Promoter

We demonstrated that Mitfa is able to activate the *sox10* promoter both by looking for *sox10* RNA in embryos after they have been injected with *mitfa* RNA and by looking for GFP in embryos from the Tg(-7.2*sox10*:GFP) line after they have been injected with *mitfa* RNA. This latter experiment showed that Mitfa binds within the 7.2 kb *sox10* promoter that was used to generate the Tg(-7.2*sox10*:GFP) line. We also identified multiple potential Mitf binding sites, in silico, within the promoter (Matys et al. 2003). However, we have neither found published evidence to suggest that Mitfa is able to activate expression of Sox10, either in zebrafish or other organisms, nor evidence that it is not able to regulate expression of Sox10. This has an interesting implication for our original model which predicted that Mitfa would repress expression of *sox10*. We now have evidence that Mitfa may be able to bind to the *sox10* promoter, perhaps in vivo this would repress *sox10* expression. A lack of specific transcription factors, or an excess of the wrong ones, in the early embryo could mean that binding of Mitfa to the *sox10* promoter will have the opposite effect in our RNA injection experiments than it would in vivo. For example, CBP/p300 is expressed in mouse melanocytes and is known to interact with Mitf, the suggestion being that recruitment of CBP/p300 assists gene activation by Mitf (Sato et al. 1997). However, zebrafish equivalents of CBP/p300 have not been described and without further understanding of these genes in zebrafish we cannot say if they may be important in regulating Mitfa activity in our experiments. Lef is another factor known to have a positive effect upon activation of genes by Mitf (Steingrímsson, Copeland and Jenkins 2004). It too is expressed in zebrafish neural crest at least at 16 hpf and is also expressed in the early embryo (Ishitani et al. 2005, Lee et al. 2006), though again it is difficult to predict the effect this might have on Mitfa target gene expression in our experiments. A third example of an Mitf co-factor is Pias3. It is known to repress activation of genes by Mitf (Sonnenblick, Levy and Razin 2004). Pias3 and other genes, such as Tfeb and Tfec, have not been described yet in zebrafish demonstrating the considerable gaps in our understanding of gene regulation by Mitfa in zebrafish and the many possible explanations for our results.

Half of the six Mitfa binding sites identified are not present in the 4.9 kb section of the *sox10* promoter used to generate another transgenic line, Tg(-4.9*sox10*:GFP) (Carney

2003). Repeating the experiments described here with this line, may shed light on which potential binding sites are likely to be functional. This could be followed up with in vitro promoter analysis (Antonellis et al. 2006) and for example, ChIP assays (Yokoyama, Takeda and Shibahara 2006), which could provide more evidence for whether the binding of Mitfa to the *sox10* promoter has a positive or negative regulatory effect in vivo.

We also found that injection of *sox10* RNA will induce expression of GFP in Tg(-7.2*sox10*:GFP) embryos. At 6 hpf, the number of embryos expressing GFP is very low, we suggest that this is because Sox10 has no effect on its own promoter. At 10.5 hpf there is an increased number of embryos expressing GFP. Sox10 can induce expression of *mitfa* and we suggest that the extra 4.5 hours between sampling times allows Mitfa to accumulate and activate expression of GFP via the transgenic *sox10* promoter. This could be tested more thoroughly by creating embryos which are both transgenic and mutant at the *mitfa* locus and repeating the experiment where we would predict that no GFP expression would now be seen.

### 5.3.3 Sox10 Can Repress Activation of Genes by Mitfa

We reproduced an experiment from Elworthy et al. (2003) where *sox10* RNA was injected into one cell stage embryos and expression of *mitfa* was found to be induced after six hours. We found that none of our other marker genes, *dct*, *silva*, *tyrosinase* or *tyrp1b*, were induced in this way suggesting that Sox10 alone is not sufficient for expression of these genes. However, *mitfa* dependent expression of *tyrp1b* was seen if embryos were allowed to develop to 10.5 hpf. This suggests that Sox10 does not directly activate expression of *tyrp1b* but that it induces expression of *mitfa*, which in turn activates the other marker genes. This was corroborated by the lack of *tyrp1b* expression when the experiment was repeated in embryos which lack functional Mitfa and fits with evidence for the regulation of Tyrp1 by Mitf in other organisms (Murisier and Beermann 2006).

Our model predicts that Sox10 is able to inhibit expression of genes downstream of Mitfa. We found that Mitfa is only able to induce expression of most of our marker genes in the initial absence of Sox10 and not when Sox10 is present in the embryo before Mitfa (i.e. after injection of *mitfa* and not *sox10* RNA). This supports our prediction and fits with data we discussed in Chapter 3 illustrating a repressive role for Sox10. We confirmed this result by injecting both *sox10* and *mitfa* RNA together into wild type embryos thus inhibiting the expression of our melanophore marker genes that would have occurred in these embryos if *mitfa* RNA had been injected alone.

However, there was an exception to this rule; again, as above, *tyrp1b* was not repressed by overexpression of Sox10. In Chapter 3 we reported that *tyrp1b* is derepressed to a lesser extent than the other marker genes in *sox10*<sup>3</sup> embryos. We suggested that this could be explained if Sox10 is less important in the repression of *tyrp1b* than in the repression of *dct*, *silva* or *tyrosinase*, or if activation of *tyrp1b* by Mitfa is less dispensable for its expression than for the expression of the other markers. The evidence presented in this Chapter supports the observation that the role of Sox10 may be less important for inhibition of *tyrp1b* expression than for regulating the other marker genes. It also suggests that factors which act either alone or in conjunction with Sox10 to inhibit *tyrp1b* expression during normal development are not present in the early embryo.

These results highlight one potential difference between our model for Sox10 function in melanophores and the model proposed by Kim et al. (2003) for its role in sympathetic neurons. In sympathetic neurons Sox10 represses expression of Phox2a, a transcription factor downstream of Phox2b and Mash1. In our model, Phox2a is represented as transcription factor X. We have no candidate for this gene and have been using our melanophore markers as downstream readouts of the transcriptional activity of X. However, our marker genes appear to behave differently from one another in these experiments and have different expression patterns in *sox10*<sup>3</sup> mutants (Chapter 3). This suggests that if transcription factor X has a role in their regulation there are also other important factors involved that differ for each gene. Alternatively, there may be no transcription factor X and *dct*, *silva*, *tyrosinase* and *tyrp1b* are regulated directly by Sox10 and independently of each other.

### 5.3.4 Levels of Sox10 are Important for its Functions

Kim et al. (2003) suggest the idea that levels of Sox10 may be important in how it regulates different genes. High levels of Sox10 are required for inhibition of Phox2a but comparatively lower levels will suffice to induce expression of Mash1 and Phox2b. We also know that haploinsufficiency for pigment phenotypes is seen in mouse *Sox10* mutants because heterozygous *Sox10* mutant mice have a pigment phenotype (Herbarth et al. 1998). This suggests that levels of Sox10 may also be important in pigment cell development. We have demonstrated that *sox10* is downregulated in developing melanophores but that it is still expressed in these cells (Chapter 4). If *sox10* always represses expression of melanophore differentiation genes then it we would not expect to see Sox10 protein in melanised cells at all. But, if high levels of Sox10 are needed for it to inhibit gene expression, only small reductions in Sox10 levels may allow expression of our marker genes and melanin synthesis. We investigated the importance of levels of Sox10 in melanophore development using our ‘living test tube’ methodology.



If we inject *mitfa* alone, levels of *mitfa* are high and all of our marker genes are expressed. Since in this case *sox10* expression is induced by *Mitfa*, there is a delay in the expression of *Sox10* and hence *Mitfa*-dependent transcription of our marker genes occurs. If we inject *sox10* RNA alone levels of *sox10* are high and *mitfa* is not expressed immediately or at high levels. This allows *Sox10* to inhibit the induction by *Mitfa* of *dct*, *silva* and *tyrosinase*. We found that if we injected *mitfa* and *sox10* RNA together into embryos, resulting in high levels of *sox10* and *mitfa* from the beginning of the experiment, then *Mitfa* induced expression of all our marker genes but *tyrp1b* was inhibited. This shows that *Sox10* is able to repress expression of *dct*, *silva* and *tyrosinase* if present early and at high levels, even in the presence of high levels of *mitfa*. To test whether the levels of *sox10* are important for it to inhibit marker gene expression, we carried out a titration experiment. Embryos were injected with mixtures of RNA containing constant amounts of *mitfa* RNA but decreasing amounts of *sox10* RNA. Generally, as levels of *sox10* decreased a higher proportion of embryos expressed each marker gene. This supports our hypothesis that high levels of *sox10* are required to inhibit marker gene expression. Since levels of *sox10* do seem to affect the function of *Sox10* it would be interesting to know how the levels we have used compare to those present in vivo. The lack of a melanophore phenotype in zebrafish *sox10* heterozygotes would suggest that half of the endogenous concentration of *sox10* is sufficient for normal melanophore development, or that the cells are able to effectively compensate for loss of one gene copy.

The data generated from this titration experiment was rather noisy. In previous experiments we have seen how variable the proportion of embryos expressing different markers can be. However, we have never seen expression of *dct*, *silva* or *tyrosinase* at the maximum *sox10* dose (Figure 5.05). In these titration experiments we do see a few embryos, sometimes many embryos, expressing our marker genes at this dose (Figure 5.07). The RNA used in the titration experiments was made on a different occasion to that used in any of the other experiments so there may have been differences in measuring RNA concentration resulting in variation in the concentrations of *sox10* RNA in each set of experiments. It may suggest that even our maximum dose of *sox10* RNA is on the borderline between being enough to repress marker gene expression and not being enough. *silva* appears to be switched on much more than the other markers even at the maximum *sox10* dose, suggesting that it may require greater concentrations of *sox10* than the other markers in order to be repressed. There were also a number of anomalous points in our graphs. There is lots of opportunity during the protocol for inconsistencies to occur, for example through pipetting inaccuracies, despite careful work and reasonable sample sizes. A separate needle was used for each RNA mixture to avoid contamination but this may have introduced differences in the quantities of

each mixture that were injected. Since levels of *sox10* seem to be important, even very subtle differences between the different mixtures or between the concentrations of DNA received by individual embryos, will introduce variability into the results.

The noise in the titration data precludes detailed observations of some of the subtle differences that appear to exist between the regulation of our different markers by Sox10. For example, *tyrosinase* expression is never seen in as high a proportion of embryos as the other markers, even at the minimum concentration of *sox10*. Given the variability in the data it is difficult to know whether this represents a real difference between *tyrosinase* and for example *dct* expression or whether it just represents experimental variability. Further to these experiments it would be interesting to inject different quantities of *sox10* alone into wild type embryos to find the lowest and highest concentrations at which Sox10 is able to repress expression of *dct*, *silva* or *tyrosinase* without affecting embryo development. Observations of marker gene expression in embryos injected with amounts of *sox10* between these two extremes together with *mitfa* RNA may give cleaner results. We could also find the lowest concentration of *sox10* required to induce expression of *mitfa*, which we would predict to be considerably lower than the lowest concentration for inhibiting marker gene expression. Similarly, the lowest concentration of *mitfa* RNA required to induce expression of our marker genes could be found. It would then be interesting to inject the lowest concentration of *sox10* needed to repress expression of our marker genes, combined with the lowest concentration of *mitfa* required to induce expression of our marker genes and explore further how the relative levels of *sox10* and *mitfa* affect the activity of each other. A second potentially useful approach that could be used would be in vitro promoter analysis. The activity of the different melanophore marker gene promoters could be measured with different quantities of Sox10 and Mitfa present in the reaction (Antonellis et al. 2006). This would have the advantage that the concentrations of Sox10 and Mitfa in each reaction could be strictly controlled but the disadvantage that other conditions would not mimic those in vivo as closely as RNA injection experiments might be expected to.

### 5.3.5 Summary

In this chapter we have shown that *mitfa* alone is able to induce expression of *dct*, *silva*, *tyrosinase* and *tyrp1b* supporting the hypothesis that in vivo, Sox10 is only necessary for expression of *mitfa* after which melanophores do not require Sox10 for their development. Mitfa was also able to induce expression of *sox10*. We found that Mitfa is able to bind within the 7.2 kb *sox10* promoter and that whilst in these experiments this induces expression of GFP/*sox10*, further work is needed to see whether endogenously it actually has a repressive role. This suggestion is plausible

since the way a transcription factor behaves in the early embryo may differ from the way it behaves later in the neural crest due to the differences in those cellular environments. A repressive role for *Mitfa* would be consistent with our original model which predicted that *Mitfa* would repress expression of *sox10*. We went on to show that whilst *sox10* is present in embryos injected with *mitfa* RNA, it is not required for the expression of our marker genes and is not able to induce expression of any of our marker genes alone (even after an extended incubation of 10.5 hours, we only see *mitfa* dependent expression of *tyrp1b*). We showed that *sox10* also is able to inhibit *mitfa* induced expression of *dct*, *silva* and *tyrosinase* and that levels of *sox10* are important in this role.

These results provide further evidence in support of our model for the role of *Sox10* in melanophore development. The model predicts that *Sox10* is only required transiently in melanophores for the expression of *Mitfa*, the expression of which is sufficient for them to develop normally. This is supported by other data in zebrafish as well as data from chick, quail and medaka (Planque et al. 1999, Béjar et al. 2003, Lister et al. 1999) but is in contrast to recent data from mouse which shows a later requirement for *Sox10* in melanocytes for the expression of *tyrosinase* and for melanocyte differentiation (Hou et al. 2006). Our results therefore support the conclusion from Hou et al. (2006) that zebrafish and mouse are likely to differ in the role that *Sox10* plays in melanophores and melanocytes. Further work in vivo in mouse and in zebrafish as well as in other organisms will show which of the two models for the role of *Sox10* in melanocytes is the paradigm.

# Chapter 6

## Discussion

### 6.1 Background

Many different genes function to regulate the development and differentiation of the neural crest and its derivatives. One such gene, which is of particular interest to us, is *Sox10*. The precise role of Sox10 in the neural crest is not completely understood, though there seem to be four main areas where it has a role. In *Xenopus* at least, Sox10 appears to play a role in neural crest induction, though this is not supported by work in other organisms (Honoré, Aybar and Mayor 2003, Aoki et al. 2003, Cheung and Briscoe 2003). In several neural crest derived cell types Sox10 has been shown to be important for regulating specification via activation of key genes such as *Mitf* in melanocytes and *Phox2b* and *ngn1* in neurons (Elworthy et al. 2005, Elworthy et al. 2003, Carney et al. 2006, Kim et al. 2003). Other evidence demonstrates that Sox10 can also function in sympathetic neurons to inhibit their differentiation and maintain cells in a stem cell-like state (Kim et al. 2003). Finally, continued expression of Sox10 is known to be important in differentiated glia for expression of genes such as *Krox20*, *MPZ* and *Connexin32* (Ghislain and Charnay 2006, Peirano et al. 2000, Bondurand et al. 2001). So, Sox10 has different functions in many different cell types, often performing different roles in each and even multiple roles within one cell type. This variety of expression and function make Sox10 an interesting and important gene to study. In this thesis, we have sought to further investigate the function of Sox10 in pigment cells, specifically zebrafish melanophores.

The role of Sox10 in melanocytes has been partially explored but current evidence has generated some controversy. In mouse, human and zebrafish Sox10 is able to initiate expression of *Mitf*, the master regulator of melanocyte differentiation. *Mitf* is able to activate expression of a wide range of melanophore specific genes including *c-Kit*, *Dct*, *Tyrosinase* and *Tryp1* and initiate melanocyte differentiation (reviewed in

Goding (2000), Steingrímsson, Copeland and Jenkins (2004)). In zebrafish, work to date suggests that the only role for Sox10 is to activate expression of *Mitfa* and that Sox10 is not required in melanophore development for any other purpose (Elworthy et al. 2003). Recent work in mouse has provided evidence to suggest that the contrary is true in this model organism; that Sox10 in mouse has an additional, later, role in melanocyte development. They found that *Mitf* alone is sufficient to activate expression of *Dct*, *Silver* and *Tyrp1* in mouse neural tube culture but that it is insufficient to activate expression of *Tyrosinase*. Further, they found that Sox10 is required in addition to *Mitf* for expression of *Tyrosinase* and proper melanocyte development. The authors suggest that this demonstrates marked interspecies differences between the role of Sox10 in mouse melanocyte development compared to its role in zebrafish melanophore development. However, we felt that insufficient evidence exists from either organism for this proposal to be considered conclusive. Data from mouse has been generated almost entirely in vitro and remains to be tested in vivo. In zebrafish, there is a lack of molecular data which is directly comparable to the mouse work. In further exploring the role of Sox10 in melanophores, we also hoped to help resolve some of the controversy surrounding this current data.

## 6.2 A Testable Model

We began with a testable model for the role of Sox10 in melanophores. This model was adapted from one proposed by Kim et al. (2003) for the role of Sox10 in sympathetic neuron development in mouse (Figure 1.04). The first relationship described by our adapted model for the role of Sox10 in melanophores is that of Sox10 directly regulating expression of *mitfa*. This had been established conclusively in zebrafish (Elworthy et al. 2003) and is well supported by data in both mouse and human (Britsch et al. 2001, Potterf et al. 2001, Bondurand et al. 2000, Lee et al. 2000, Potterf et al. 2000, Verastegui et al. 2000). In zebrafish, it was demonstrated that *sox10* mutants lack expression of *mitfa* and that overexpression of *sox10* RNA in the embryo will induce expression of *mitfa*. Further, Sox10 can activate expression from the *mitfa* promoter via Sox10 binding sites within that promoter (Elworthy et al. 2003). From our model we then made several predictions. First, that Sox10 would also, simultaneously to activating *mitfa*, repress expression of melanophore differentiation genes. Following on from this, we predicted that Sox10 would be downregulated in differentiating melanophores and expected that this downregulation would be necessary for melanophores to differentiate. The model also predicts that *Mitfa* will be responsible for the downregulation of Sox10. We were able to test most of these predictions during our work presented here.

### 6.2.1 Mitfa Regulates Melanophore Specific Genes

Work from Elworthy et al. (2003) suggests that the only necessary role that Sox10 performs in melanophores is to activate expression of *mitfa*. Overexpression of *mitfa* in the neural crest of embryos which are mutant for *sox10* or *mitfa*, rescued similar numbers of melanophores in the same proportion of embryos of either genotype. Work in mouse fibroblasts and in chick, zebrafish and medaka found Mitf to be sufficient to drive expression of melanogenic enzymes and melanogenesis in these models (Lister et al. 1999, Tachibana et al. 1996, Béjar, Hong and Scharl 2003, Planque et al. 1999), supporting the hypothesis that Mitf alone is sufficient for melanocyte differentiation. In addition, a great deal of evidence exists from both mouse and human that shows Mitf is able to activate expression from *Tyrosinase* (Yasumoto et al. 1997, Yasumoto et al. 1994, Bentley, Eisen and Goding 1994, Ganss, Schütz and Beermann 1994), *Tyrp1* (Bertolotto et al. 1998, Yasumoto et al. 1997, Lowings, Yavuzer and Goding 1992, Jackson et al. 1991), *Dct* (Budd and Jackson 1995, Jackson et al. 1992, Bertolotto et al. 1998, Yasumoto et al. 1997, Yokoyama et al. 1994) and *Silver* (Du et al. 2003) promoters. However, there is also evidence to suggest that Mitf may not be sufficient for melanocyte differentiation. Mitf expressed alone in mouse neural tube explants is not sufficient to drive expression of *Tyrosinase* and development of pigmented melanocytes (Hou, Arnheiter and Pavan 2006). In this case, Sox10 is required in addition to Mitf for *Tyrosinase* expression. Sox10 is also implicated in the expression of *Dct* in that it can bind to (Britsch et al. 2001, Potterf et al. 2001), and activate expression from (Jiao et al. 2004, Ludwig, Rehberg and Wegner 2004), the *Dct* promoter in mouse. However, Hou et al. (2006) demonstrated that whilst Sox10 may be able to activate *Dct* expression it is not necessary for *Dct* expression.

Our model implicitly predicts that Mitfa will be sufficient to drive expression of melanophore differentiation genes and it was necessary to test this prediction before we could test whether Sox10 was able to inhibit expression of the same genes. We injected *mitfa* RNA or *mitfa*<sup>w2</sup> RNA into one cell stage *sox10*<sup>3</sup> mutant embryos and found that *mitfa*, but not *mitfa*<sup>w2</sup>, induced detectable expression of *dct*, *silva*, *tyrp1b* and *tyrosinase* six hours after injection. This evidence shows that Mitfa can induce expression of all of our marker genes. In support of this conclusion we were able to demonstrate again, using a similar method, that *tyrp1b* expression is Mitfa dependent. Injection of *sox10* RNA into wild type embryos will induce *mitfa* expression after six hours and *tyrp1b* after 10.5 hours, whereas induction of *tyrp1b* expression in this way fails in *mitfa*<sup>w2</sup> mutants. This demonstrates that Sox10 activates expression of *mitfa* in wild type embryos and Mitfa in turn activates expression of *tyrp1b*. This explains the delay in *tyrp1b* expression as compared to *mitfa* expression after injection of *sox10* RNA into wild type embryos. Further, our evidence suggests that Mitfa



may not simply induce expression of *dct*, *silva*, *tyrp1b* and *tyrosinase* but that it may induce expression of these genes directly. We know that activation of *mitfa* by Sox10 is direct and that *mitfa* mRNA is present six hours after injection with *sox10* RNA; we see similarly rapid activation of our marker genes in embryos after injection with *mitfa* RNA suggesting a similarly direct relationship.

Ideally, further experiments would be carried out to confirm our findings. To demonstrate that marker gene expression after injection with *mitfa* RNA was Sox10 independent we injected *mitfa* RNA into embryos for a heterozygous *sox10*<sup>3</sup> incross. In the cases of *dct*, *silva* and *tyrp1b* the results were clear: significantly more than 75 % of embryos in each case expressed the marker gene so we knew that even embryos without functional Sox10 were able to express each gene. The case was less clear cut for *tyrosinase* where the number of *tyrosinase* expressing embryos was not significantly different from 75 %. However, induction of our markers was not fully penetrant even in wild type embryos where only 85 % of injected embryos expressed *tyrosinase*. We felt that the 27 % of embryos that did not express *tyrosinase* was not enough to represent the 25 % of embryos that should be mutant plus a large enough percentage of wild type embryos that we also expected not to express *tyrosinase*. Ideally we would have liked to genotype these embryos to definitively verify our result but we were unable to do so. This was partly due to the poor quantity and quality of DNA obtainable from young embryos which had been processed for ISH. However, this was exacerbated by the nature of the mutation in *sox10*<sup>3</sup>, a fairly large transposon insertion in the *sox10* gene which also contains repeat elements (Carney 2003). This makes it difficult to genotype even untreated embryos at 3 dpf (T. Carney, personal communication). An alternative approach that may prove easier would be to optimise primers and PCR conditions to genotype embryos from a different *sox10* mutant, for example *sox10*<sup>m618</sup> which has a point mutation. The experiment could then be repeated in this line to fully verify our result.

Our results outlined above support the prediction that Mitfa is sufficient, at least does not require Sox10, to induce expression of all of the genes we studied and leaves open the possibility that it is sufficient to induce many other melanophore differentiation genes. However, this does not exclude the possibility that other proteins may be required for the induction of melanophore specific genes by Mitfa or to enhance the induction of those genes. For example, Mitfa will often work as a heterodimer with various partner proteins (see 1.2.1i), so perhaps there are co-factors present in the early embryo that are equivalent to ones that would normally exist in the developing neural crest, and are needed to allow Mitfa to induce gene expression in our experiments. For example, Lef1 might be such a co-factor and we know that it is expressed ubiquitously at the 4

cell stage when it could alter the results from our RNA injection experiments and in the neural crest at 18 hpf where it might function in normal neural crest development (Dorsky et al. 1999). If other factors are required for Mitfa-dependent gene expression, then whilst Mitfa may be necessary it may not be entirely sufficient to induce target gene expression. This would be difficult to test using our methodology but may be testable using in vitro transcription from, for example, the *dct* promoter. Looking for changes in levels of transcription in the presence and absence of Mitfa and various possible co-factors such as Tfe3 or Lef1 would differentiate between Mitfa being necessary or sufficient for melanophore marker gene expression. What we have been able to show is that Mitfa can induce target gene expression in zebrafish even in the absence of functional Sox10. This is contrary to evidence from mouse which suggests that Mitfa activation of *Tyrosinase* is Sox10 dependent. Thus, if we ignore the weaknesses in the mouse data as data collected primarily in vitro, our results add support to the conclusion drawn by Hou et al. (2006) that there are significant interspecies differences between the regulation of melanocyte development in mouse and zebrafish.

At this point it is interesting to note a difference between our model and that proposed by Kim et al. (2003) for sympathetic neuron differentiation. In their model, Sox10 promotes expression of two transcription factors, Mash1 and Phox2b. Downstream of these two genes is Phox2a, another transcription factor. In our model, we know that Sox10 promotes expression of Mitfa and we might expect that downstream of Mitfa there will be another transcription factor, transcription factor X. The only transcription factor that we can identify that has been proposed to be downstream of Mitf is an example from mouse. Tbx2 is a T-box transcription factor which is not expressed in melanoblast precursor cells but is expressed in melanoblasts and melanocytes (Carreira et al. 1998). The same group who cloned and described the expression of Tbx2 went on to demonstrate that the *Tbx2* promoter contains an Mitf recognition sequence to which Mitf binds in vitro. Further, Mitf is able to activate expression from the *Tbx2* promoter in cell culture (Carreira, Liu and Goding 2000). This evidence makes Tbx2 an excellent candidate for transcription factor X in our model. However, zebrafish have two *Tbx2* genes, *tbx2a* and *tbx2b*, neither of which has been shown to be expressed in the neural crest or neural crest derived melanocytes (Thisse and Thisse 2004). In addition, as described above, there is a great deal of evidence to suggest that Mitfa is itself able to directly regulate melanophore differentiation genes such as *dct*. It would be valuable to explore this possibility further in zebrafish by analysing the promoters of our melanophore marker genes in search of Mitfa responsive M-boxes for example. Even without this evidence however, we feel able to suggest that there may be no transcription factor X in melanophores. Our model for the role of Sox10 in melanophore development is therefore simpler in this respect than that for its role in neuron development.

### 6.2.2 Sox10 Inhibits Melanophore Specific Genes

We have established that *Mitfa* is able to induce expression of *dct*, *silva*, *tyrp1b* and *tyrosinase*. Our model predicts that Sox10 will have an inhibitory effect upon the expression of these genes. Two kinds of experiment provided data testing this part of the model. In the first set of experiments, we made observations of the expression of endogenous *dct*, *silva*, *tyrosinase*, *tyrp1b* and melanin in *sox10<sup>l3</sup>*, *mitfa<sup>w2</sup>* and *sox10<sup>l3</sup>;mitfa<sup>w2</sup>* mutants. We saw that in *mitfa<sup>w2</sup>* embryos, where Sox10 is still expressed but *Mitfa* is not functional, all of our melanophore markers were expressed. However, expression was extremely weak and was only seen in a very small number of cells in a premigratory neural crest position; indeed *tyrp1b* was hardly expressed at all. This was consistent with a lack of any residual melanised cells in these mutants. In *sox10<sup>l3</sup>* embryos, where Sox10 is not functional and *mitfa* is not expressed, our markers were expressed more strongly and in more cells than in *mitfa<sup>w2</sup>* embryos, though expression was still weak when compared to wild type expression. However, now we were also able to observe residual cells which were able to express melanin, presumably as a result of the stronger residual expression of our melanophore marker genes. Observations in *sox10<sup>l3</sup>;mitfa<sup>w2</sup>* embryos confirmed that double mutants had the same phenotype as *sox10<sup>l3</sup>* homozygous embryos indicating that the residual expression seen was *mitfa* independent. From these observations we concluded that expression of *dct*, *silva*, *tyrosinase* and *tyrp1b* is derepressed in the absence of Sox10. Thus, in wild type embryos we suggest that Sox10 would normally function to repress expression of those genes and inhibit melanophore development. This work does not test whether Sox10 directly binds to and inhibits expression from the various marker gene promoters. It could be that Sox10 indirectly mediates repression by promoting expression of other factors which, in turn, repress expression. Of our markers, only *Dct* expression has been looked at in *Sox10* mutant mice. Here *Dct* expression was lost altogether suggesting that similar derepression of melanocyte markers may not occur in the absence of Sox10 in mouse (Britsch et al. 2001, Potterf et al. 2001, Southard-Smith, Kos and Pavan 1998). However, this could be an artefact of the relatively poor sensitivity of whole mount ISHs in mouse. It would be interesting to look for *Silver* expression in *Sox10* mutant mice as zebrafish expression of *silva* appears stronger than the other markers and therefore, if it is derepressed *Silver* might be easier to detect in mouse. Careful RT-PCR analysis excluding material from parts of the embryo which are not affected by loss of Sox10, such as the PRE, might also reveal more.

Our second approach to exploring some of the genes Sox10 may be able to regulate in zebrafish melanophores involved RNA injection into one cell stage embryos and assaying by ISH for induced gene expression. Despite evidence from mouse which implies that Sox10 is able to induce expression of *Tyrosinase* and *Dct* (Hou et al. 2006,

Britsch et al. 2001, Potterf et al. 2001, Jiao et al. 2004, Ludwig et al. 2004) we found no evidence to suggest a direct positive relationship in zebrafish between Sox10 and any of the melanophore markers we used. We have shown that injection of *mitfa* RNA robustly induces expression of all of our marker genes and we were still able to induce expression of all of our markers by injection of *mitfa* into *sox10*<sup>3</sup> mutant embryos. Whilst this does not show that Sox10 is not able to positively regulate these genes, it does suggest that it is not required for their induction. In fact we have generated evidence to suggest that Sox10 may actually have a negative effect on melanophore marker gene expression. We know that Sox10 is able to induce expression of *mitfa* (Elworthy et al. 2003) and that Mitfa is able to induce expression of melanophore marker genes. Therefore, if Sox10 has no inhibitory effect on marker gene expression, injection of *sox10* RNA should result in expression of *mitfa* and of our marker genes. We were able to induce expression of *mitfa* at 6 hpf by injection of *sox10* RNA but of our four marker genes none were expressed at 6 hpf and only *tyrp1b* was expressed at 10.5 hpf. It could be that levels of induced Mitfa in this experiment only get high enough to induce expression of *tyrp1b* and that lower levels are not sufficient for *dct*, *silva* and *tyrosinase* expression. However, we found that *mitfa* RNA injected with *sox10* RNA still only resulted in induced expression of *tyrp1b* suggesting that this is not the case. We concluded that under the conditions used, Sox10 is able to inhibit Mitfa-dependent expression of *dct*, *silva* and *tyrosinase*. The rapidity with which Sox10 is able to inhibit expression of our marker genes suggests direct relationships between Sox10 and the marker genes. This remains to be tested but could provide some interesting data about the various promoters. It would be valuable to analyse the promoter sequences of our marker genes and discover whether there are Sox10 binding sites present within them. ChIP assays could be used to determine whether Sox10 does in fact bind to these sequences. Subsequent in vitro work using the promoters to drive reporter gene expression might confirm whether Sox10 exerts a positive or negative regulatory effect on the promoters and determine the effect of Sox10 on Mitfa dependent promoter activation.

Further investigation of the different promoters would be interesting for a second reason. Each of our different markers showed differences in their expression patterns in *sox10*<sup>3</sup> and *mitfa*<sup>w2</sup> embryos. This is most striking in the case of *tyrp1b* where expression was considerably weaker than that of the other markers in *sox10*<sup>3</sup> embryos and almost entirely absent from *mitfa*<sup>w2</sup> embryos. This suggested that *tyrp1b* expression may be more dependent upon *mitfa* expression than the other genes are and/or that factors other than Sox10 are more important in repression of its expression than for the repression of our other markers. Supporting this suggestion, we also found a lack of repression of *tyrp1b* by Sox10 in our RNA injection experiments. Rather than the effect of Sox10

repression only being weaker for *tyrp1b* than the other markers, as we saw in mutant embryos, here we saw an apparently complete lack of *tyrp1b* repression by Sox10. This difference in effects is probably due to differences between the environment of the early embryo as compared to that in the developing neural crest. For example, if factors other than Sox10 are important for *tyrp1b* repression these genes are likely to still be expressed in the neural crest of *sox10<sup>l3</sup>* embryos and maintain some repression of *tyrp1b*. However, it is possible that they will not be expressed in the early embryo to repress *tyrp1b* expression in our RNA injection experiments so that we see *tyrp1b* expression after injection of *sox10* RNA.

*silva* also has a particularly striking expression pattern, particularly in *sox10<sup>l3</sup>* embryos, in that it is expressed in more cells which extend over a larger area of the embryo than the other markers. Expression of *silva* also appears to persist slightly longer so that more embryos at later stages still express it. This is in contrast to *tyrp1b* expression and suggests that the relative importance of different factors in *silva* regulation will contrast with those important in *tyrp1b* regulation. Our results suggest that *Mitfa* is less important for driving *silva* expression than for driving expression of the other markers. They also suggest that Sox10 may be more important than other factors to repress *silva* expression. *tyrosinase* differs from the other markers in that it is expressed more strongly in *mitfa<sup>w2</sup>* embryos than are the other markers. *tyrosinase* is expressed more strongly and over a longer time period in *sox10<sup>l3</sup>* as compared to *mitfa<sup>w2</sup>* embryos, so it is still likely that we are observing derepression of *tyrosinase* in the absence of Sox10. The strength of expression in *mitfa<sup>w2</sup>* mutants suggests that Sox10 is less able to repress expression of *tyrosinase* than it is able to repress expression of the other markers. However, if this were the case we might expect *tyrosinase* to be expressed more than the other markers in *sox10<sup>l3</sup>* embryos, which it is not. This implies that there may be another layer of complexity in the regulation of *tyrosinase* expression not seen for the other markers. The data described thus far would fit with a model whereby Sox10 is able to repress expression of *tyrosinase* but it also indirectly promotes *tyrosinase* expression via both factor Y and *Mitfa*. In *sox10<sup>l3</sup>* embryos Sox10 is not expressed so neither is factor Y and consequently *tyrosinase* is derepressed. In *mitfa<sup>w2</sup>* embryos Sox10 is expressed so factor Y would also be expressed and be able to promote *tyrosinase* expression despite the lack of functional *mitfa* and the fact that Sox10 is also weakly repressing such expression. Thus, we see more *tyrosinase* expression in these embryos than we would predict if factor Y were not involved.

We know that *sox10<sup>l3</sup>* embryos do not express *mitfa* and by looking at *sox10<sup>l3</sup>;mitfa<sup>w2</sup>* embryos we have shown that the expression of marker genes seen in these embryos is



*mitfa* independent. Yet we do still see expression of our markers in *sox10*<sup>3</sup> mutants. Therefore, we suggest that there is a pathway by which these genes can be activated which does not involve *Mitfa*. We observed that residual cells expressing marker genes or melanin develop in an anterior to posterior fashion, much as they would in a wild type embryo. This suggests that the means by which our marker genes are activated in mutant embryos may play a role in normal development too. Without *Mitfa* expression, activation of our marker genes is very weak and we know that other genes important for melanophore survival, such as *c-kit*, cannot be detected by in situ hybridisation (Dutton et al. 2001). The means by which our marker genes are activated in mutant embryos must be less efficient in the absence of *Sox10* and *Mitfa* and is not sufficient for expression of all important melanophore genes. Perhaps another transcription factor is involved which acts synergistically with *Mitfa* to enhance target gene expression well above what either factor could achieve individually. For example, *Mitf* and *Lef-1* can act synergistically to activate high levels of expression of *Dct* in cell culture when individually activation is only weak (Yasumoto et al. 2002). Thus there is the possibility that *Lef1* could activate expression of *dct* in *sox10*<sup>3</sup> embryos despite the lack of *Mitfa*. Indeed, *lefl* is expressed in the zebrafish neural crest at 18 hpf and in early development (Dorsky et al. 1999). Later stages have not been examined so we cannot know whether expression persists. Nevertheless, expression at 18 hpf is appropriate for activating melanophore marker genes and so *Lef1* remains a candidate for promoting melanophore marker gene expression in the absence of *Mitfa*.

Our observations of marker gene expression in *sox10*<sup>3</sup> mutants also support previous data about the fate of neural crest cells in these embryos. Data from zebrafish and mouse show that there is increased cell death in the neural crest of *sox10* mutants (Dutton et al. 2001, Kapur 1999, Southard-Smith et al. 1998). They suggest that crest cells which fail to specify die by apoptosis. We observed a decrease in the number of cells positive for melanophore marker genes from approximately 36 hpf. This is consistent with the timing of cell death in zebrafish observed in single cell labelling and TUNEL experiments. Our close observations of the residual melanised cells in *sox10*<sup>3</sup> mutants showed the usual morphology of these cells to be small, punctuate and blebbed. We also observed the collapse of a larger cell to adopt this apoptotic morphology. These observations are consistent with loss of unspecified neural crest cells by apoptosis, though it is interesting to note that these unspecified cells have been able to produce melanin, a characteristic of differentiating melanophores. It would be interesting to look for co-localisation of TUNEL labeling and residual pigment to confirm that residual pigmented cells are the remains of apoptosed pigmented crest cells.



### 6.2.3 Sox10 and Xanthophore Development

Melanophores are not the only pigment cells which seem to partially differentiate in *sox10*<sup>3</sup> embryos; xanthophores can also be observed in these embryos though they are abnormal and disappear between 35 hpf and 45 hpf (Dutton et al. 2001). Based on this observation we wondered whether Sox10 might perform similar roles in both melanophores and xanthophores and therefore whether we might see residual xanthophore gene expression in *sox10*<sup>3</sup> embryos. We looked at expression of xanthophore markers *gch*, *xdh* and *paics* in *sox10*<sup>3</sup> embryos and found that we could observe residual expression of all of the xanthophore markers we used in *sox10*<sup>3</sup> mutants. As with the melanophore markers, the xanthophore markers were expressed in cells in a premigratory neural crest position. We suggest that the similarities in the expression patterns of these two sets of markers shows that Sox10 may have a similar role in xanthophores as melanophores, to repress differentiation genes and development. This possibility is more difficult to investigate in xanthophores than melanophores because far less is known about xanthophores. For example, there is no clear candidate for a master regulator of xanthophore development, i.e. a xanthophore equivalent of Mitfa. Recent work has demonstrated that both Pax3 and Pax7 are key genes in xanthophore development (Minchin and Hughes 2008). Whilst Pax7 is expressed in xanthophores it is expressed later than xanthophore markers such as *xdh*, indicating that it is not required for xanthophore specification. However, morpholino knockdown of Pax3 results in a loss of cells expressing a number of xanthophore markers and a loss of xanthophores, suggesting that it is a xanthophore specification factor. However, knockdown of Pax3 also disrupts enteric neuron development and Minchin et al. (2008) also found that whilst loss of Pax3 does not disrupt *sox10* expression prior to 18 hpf, it does disrupt *sox10* expression from approximately 25 hpf. This suggests that the role of Pax3 in the neural crest is more complex than that of Mitfa since it is required for maintenance of *sox10* expression in late neural crest. This makes it unlikely that Pax3 is a master regulator of xanthophore development in quite the same way as Mitfa is in melanophore development. It would be interesting to examine the expression pattern of *pax3* in *sox10*<sup>3</sup> mutants to learn more about the relationship between *sox10* and *pax3* and whether Sox10 also influences Pax3 expression. Sox10 and Pax3 have been shown to be able to function together to drive *mitfa* expression in vitro (Bondurand et al. 2000, Potterf et al. 2000), perhaps they have dual functions, both to regulate each other and to drive expression of xanthophore differentiation genes such as *gch* and *xdh* together. However, it also remains possible that an as yet undiscovered xanthophore specific transcription factor exists downstream of both Sox10 and Pax3 so that they indirectly influence the expression of xanthophore differentiation genes.

Additional complexity is added to the xanthophore story because expression of the

most broadly expressed xanthophore marker, *gch*, is known to overlap with expression of melanin in wild type embryos and *xdh* expression overlaps with *mitfa* expression (Parichy et al. 2000b). So, are the *gch* and *xdh* expressing cells in *sox10*<sup>-/-</sup> embryos melanoblasts, xanthoblasts or perhaps a pigment cell precursor which has been unable to become specified as one cell type and so expresses genes indicative of more than one? *gch* and *silva* have particularly broad residual expression domains in *sox10*<sup>-/-</sup> embryos and it seems unlikely that they do not overlap. This provides some, weak, support for the idea of a pigment cell precursor. Further work to confirm that *paics* expression does not overlap with either *mitfa* or melanin expression would be important to provide a definitive xanthophore marker. This could then be followed by a series of double ISHs with melanophore and xanthophore markers in *sox10*<sup>-/-</sup> embryos. Although this might prove difficult due to the low levels of residual expression in these embryos, it could identify cells which express markers indicative of both cell types. Such cells might represent pigment cell precursors that are normally only present transiently in wild type embryos but which, in the absence of Sox10, are unable to differentiate further and get stuck in this precursor state. Alternatively such cells may never be present in wild types and are only observed here because crest cells have developed entirely abnormally in the absence of functional Sox10.

Our own work provides another clue that the residual cells in *sox10*<sup>-/-</sup> embryos may have more potential to differentiate into multiple cell types than was previously anticipated and therefore could represent a pigment cell precursor. We were able to drive expression of *sox10* from a *Dct* promoter in *sox10*<sup>-/-</sup> embryos and rescue all kinds of pigment cells. One might expect that cells which are expressing *dct* are specified as melanoblasts. However, this experiment suggests that in *sox10*<sup>-/-</sup> mutants these cells are not committed to one fate and are still flexible enough to generate other pigment cell types if their situation changes, i.e. if they begin to express *sox10*. This is consistent with the fact that neural crest cells appear to fail to become specified; in the case of the melanophore this means failing to express *mitfa*, and die by apoptosis (Dutton et al. 2001). This is despite the fact that, as we have described, these cells are able to express several melanophore markers and produce melanin. This work suggests that Sox10 maintains cells in a multipotent state but that Sox10 is also required to ensure that cells become properly committed to the melanophore fate. This would be consistent with our model for the role of Sox10 in melanophore development and with the model proposed by Kim et al. (2003) for its role in sympathetic neurons. It would be interesting to repeat the experiment driving *sox10* expression in the *Dct*-expressing crest cells of *sox10*<sup>-/-</sup> mutants and looking for other neural crest derivative markers to determine whether this phenomenon extends beyond pigment cells. Can we rescue other neural crest derivatives such as enteric neurons by expressing *sox10* in this way?

This may provide some interesting insight into neural crest development in wild type embryos. If we found that the *dct* expressing cells in a *sox10*<sup>3</sup> mutant are restricted to form pigment cells, this would be supportive of a progressive fate restriction model of neural crest development (Le Douarin and Dupin 2003). It might also help us to think about questions such as ‘When does a cell become committed to a particular cell type?’ ‘Is a wild type cell expressing *dct* still malleable in its final fate?’

The results and ideas described above are reminiscent of recently published data about zebrafish *ltk*. *ltk* is expressed transiently in the neural crest between 18 hpf and 28 hpf and then from 30 hpf onwards in iridophores (Lopes et al. 2008). It is proposed that *ltk* has a role in the specification of iridophores (Lopes et al. 2008). More interesting in terms of our work, Lopes et al. (2008) also looked at the expression of *ltk* in *sox10* mutants. They found that in these mutant embryos *ltk* was expressed in an increased number of cells in a premigratory neural crest position. The pattern is remarkably similar to that which we see for our melanophore and xanthophore markers. Lopes et al. (2008) showed that the *ltk* expression domain was more anterior than that of early crest markers such as *snail2* and *foxd3* and therefore that *ltk* expressing cells could no longer be described as early crest but were actually developmentally older cells. Whilst in wild type embryos the expression of *ltk* and *sox10* do not usually overlap, in *sox10* mutants many cells can be observed which express both *sox10* and *ltk*. The authors suggest that these cells may be multipotent pigment cell precursors caught in what would normally be a transient state. It would be extremely interesting to perform further double ISHs, in various combinations, to determine whether *ltk*-positive cells are also positive for melanophore and xanthophore markers and to discover if expression of these markers ever overlaps with *sox10* expression in *sox10* mutant embryos. If this were found to be the case it would provide further support for the concept of a multipotent precursor which is able to give rise to all the different pigment cells.

#### 6.2.4 Sox10 is Downregulated in Melanophores

We have been able to show that Sox10 can repress expression of melanophore marker genes in RNA injection experiments and that the same genes are derepressed in embryos which lack functional Sox10. This supports our model which predicts that in vivo, Sox10 is able to inhibit melanophore development. Therefore, for development to proceed, our model predicts that Sox10 will have to be downregulated. Previous studies had suggested that *sox10* mRNA is lost from differentiating cells (Dutton et al. 2001) and that the *sox10* promoter is switched off in melanophores as they develop (Carney 2003). Our data show that both Sox10 protein and *sox10* mRNA are indeed switched off in melanophores as they differentiate. This verifies earlier observations,

provides a more detailed picture of Sox10 downregulation in melanophores and provides evidence in support of our model. We found that Sox10 and *sox10* begin to be lost from approximately 33 hpf. *sox10* is lost rapidly and at a constant rate from pigmented melanophores. Sox10 is only lost slowly at first suggesting that cells might first lose *sox10* expression and then lose Sox10 protein expression. However, loss of Sox10 then accelerates until eventually very few, and finally no pigmented cells can be observed expressing it. The pattern of loss of *sox10* is consistent with simple degradation of the mRNA if it is not being replaced by subsequent transcription of the gene. The sigmoid curve seen for loss of Sox10 expressing melanophores is more consistent with controlled breakdown of the protein in the cells. This could suggest that the timing of loss of Sox10 is important. Perhaps Sox10 must be present long enough to ensure that it has induced high enough levels of Mitfa but not so long that it inhibits differentiation longer than is necessary. Alternatively, perhaps Sox10 is used to delay differentiation in particular cells until it is appropriate for them to differentiate. We did not observe anything to suggest that Sox10 is shuttled between the nucleus and the cytoplasm, as has been described in mouse cell culture (Rehberg et al. 2002).

Data from other organisms does not always correlate with our own. As we would expect if our model extends to the role of Sox10 in human melanocytes, melanocytes from primary human skin cultures do not express Sox10 (Cook et al. 2005). However, Sox10 is expressed in mouse melanocyte and melanoma cell lines (Southard-Smith et al. 1998, Khong and Rosenberg 2002, Kamaraju et al. 2002) and in mouse melanocytes in vivo from embryonic stages until at least P6 (Osawa et al. 2005). Sox10 expression is lost from melanocyte stem cells however. This supports other work in mouse that suggests Sox10 may be important for melanocyte differentiation and that it may need to be lost for cells to maintain a stem cell-like state in this model organism (Osawa et al. 2005, Hou et al. 2006). This is quite opposite to the role that we suggest it plays in zebrafish melanophores. However, current data from mouse is not exhaustive since results have usually been obtained as a by-product of working to answer different questions. In addition, much of the work has been carried out in vitro and in immortal cell lines with all of the caveats that this entails. We have endeavoured to provide comprehensive in vivo data about Sox10 expression in zebrafish melanophores. We suggest that it would not be wise to draw firm conclusions about similarities and differences between zebrafish and mouse until similar studies have been conducted in vivo in mouse. Such a study should be specifically designed to document *Sox10* and Sox10 expression in all kinds of mouse melanocytes over an extended timecourse from melanoblast to pigmented melanocyte at closely spaced intervals.

It is perhaps surprising that Sox10 is expressed at all in pigmented zebrafish melanophores given that we have shown it is able to inhibit melanophore gene expression. Kim et al. (2003) were able to demonstrate that levels of Sox10 alter its ability to perform its different functions. In sympathetic neurons, whilst low levels of Sox10 are enough to initiate expression of *Mash1* and *Phox2b* they are not enough to repress expression of *Phox2a*. Kim et al. (2003) saw comparatively normal expression of *Mash1* and *Phox2b* in *Sox10* heterozygotes but found that *Phox2a* was precociously expressed. We predicted that a similar mechanism might be at work in melanophores so that initially, high levels of Sox10 promote expression of *mitfa* and inhibit expression of melanophore marker genes. As Sox10 is downregulated there is still high enough levels to maintain expression of *mitfa*, although this may no longer be necessary. However, levels may now be too low to maintain repression of melanophore markers and we begin to see expression of these genes and overt differentiation despite the persistent Sox10 expression that we have observed in pigmented cells. Our immunofluorescence results are not quantitative and do not enable us to test this hypothesis. Nonetheless, we were able to use a different technique to begin to test this hypothesis. We injected *mitfa* RNA together with decreasing quantities of *sox10* RNA and found that at lower levels, *sox10* was no longer able to repress Mitfa induced expression of our marker genes. It would be important to push this experiment further to really test our hypothesis. For example, it would be valuable to find the minimum *sox10* dose required to repress expression of marker genes. We would expect this to be higher than the minimum dose required to initiate *mitfa* expression.

Having established that Sox10 is downregulated in melanophores as they differentiate it became important to test whether this downregulation was necessary for melanophores to differentiate. We set about making a transgenic line called Tg(Dct;CFP-sox10) which used 3.6 kb of the mouse *Dct* promoter to drive expression of a *CFP-sox10* fusion in melanophores. We were able to successfully generate a construct for this purpose and demonstrate that the *CFP-sox10* fusion was functional by rescuing pigment cells in *sox10*<sup>-3</sup> mutants. We went on to produce 11 different lines from our construct. CFP was visible in the founders and in the F1 generations of these lines. Unfortunately, CFP was not visible in the F2 generations of these lines and from this we had to conclude that the whole *CFP-sox10* fusion was not expressed at significant levels, rendering these lines useless for our intended experiment. The hypothesis that Sox10 downregulation is necessary for melanophore differentiation therefore remains to be tested. As we discussed in Chapter 4, this could be achieved using transgenic approaches or by in vitro analysis to test the activity of the promoters for our marker genes with different concentrations of Sox10 and Mitfa.

We cannot give a definitive explanation as to why visible CFP was lost in the F2 generation of our Tg(Dct;CFP-sox10) fish. One possibility was that the transgene was somehow destroyed or lost from the genome, by chromosomal rearrangements for example. However, we were able to rule this out by positively genotyping multiple fish from the F1 generation as containing the transgene. Whilst F2 progeny from these fish were negative for CFP by visual inspection, they too tested positive for our transgene. Another possibility was that since our promoter was not an endogenous zebrafish promoter it was targeted for silencing. We also feel that we can rule out this and any other effects which might have occurred due to the promoter we used because an equivalent number of lines were generated which expressed only CFP from the same mouse *Dct* promoter and none of these display silencing in the F2 generation. Some recent work has described silencing of ubiquitous zebrafish promoters in particular organs but this differs from our transgenics in that silencing occurs in earlier generations and is organ specific (Thummel, Burket and Hyde 2006). We hypothesise that the explanation for the silencing we have observed could lie with the *CFP-sox10* fusion part of the construct. Apart from the obvious variability in integration sites in each individual line, this is the only consistent difference between our Tg(Dct;CFP-sox10) and Tg(Dct;CFP) lines and we did not observe silencing of any of our Tg(Dct;CFP) lines. Alternatively, we also found that vector sequences were incorporated into the genomes of Tg(Dct;CFP-sox10) fish but not in any of the five Tg(Dct;CFP) fish we genotyped. This vector incorporation was unexpected and this could also be linked to the transgene silencing that we have observed.

Despite the disappointment with the Tg(Dct;CFP-sox10) lines, we did successfully generate several useful lines which express CFP primarily in melanoblasts, Tg(Dct;CFP). These lines could be useful tools for the study of melanophore development for anything from sorting melanophores from embryos to tracking cells in vivo and in vitro. In addition, one of the lines had an interesting, ectopic, expression pattern. In this line CFP was expressed exclusively in xanthophores. Given the lack of xanthophore markers described, further characterisation of this line may provide another useful tool, this time for the study of xanthophores.

### 6.2.5 *Mitfa* Can Regulate *sox10*

The final prediction from our model was that *Mitfa* would be the transcription factor that downregulates *Sox10*. We had initially intended to test this prediction by overexpressing *mitfa* RNA in embryos and looking to see whether this delayed *sox10* expression. However, in other experiments exploring which melanophore specific genes *Mitfa* can regulate we found that injection of *mitfa* RNA into one cell stage embryos actually induced expression of *sox10*. We went on to demonstrate in similar



experiments that *Mitfa* can activate expression from 7.2 kb of the zebrafish *sox10* promoter. In silico investigation of that 7.2 kb section of the zebrafish *sox10* promoter revealed six potential *Mitf* binding sites within the promoter. There is no evidence for any positive feedback relationship between *Sox10* and *Mitf* in the literature, perhaps largely because such a relationship has not been looked for. Yet, the data presented here certainly suggest that *Mitfa* is able to regulate *Sox10*. A positive relationship whereby *Mitfa* induces expression of *sox10*, however, seems unlikely as we would expect this to result in ever increasing levels of *sox10* and *mitfa* in cells when we know that *sox10* is downregulated as melanophores differentiate. We suggest that in the neural crest in vivo, *Mitfa* may not positively regulate *sox10*, as has been demonstrated in our RNA injection experiments, but that in the neural crest this is a negative relationship, as predicted by our model. The early embryo is a very different environment to the developing neural crest and this could alter the way that different transcription factors perform. If particular factors were missing from or present in the early embryo as compared to the neural crest, the function of *Mitfa* could be altered so that whilst it may repress *sox10* expression in the crest, it is able to activate *sox10* expression in the early embryo. It would be very interesting to dissect the *sox10* promoter and determine which the important *Mitfa* binding sites are. A starting point would be to inject *mitfa* RNA into Tg(-4.9*sox10*:GFP) embryos and look for GFP induction. The *sox10* promoter used in this transgenic line has only some of the *Mitfa* binding sites that are present in the line we used originally and so this experiment might narrow down which binding sites are important. In vitro dissection of the promoter will narrow it down further and should provide more information as to whether *Mitfa* does indeed repress or promote expression of *sox10*.

### 6.2.6 Pax3

We have begun to explore a model for how *Sox10* might function in zebrafish melanophore development. We have considered a very simplified model with a limited number of variables. As we begin to learn more about this simplified network it becomes appropriate to expand it and to look at how other factors might fit into the network. For example, *Pax3* is a paired box transcription factor and mutations in this gene are associated with WS1 and WS3 resulting in similar symptoms as do mutations in *SOX10* and *MITF* in humans (Tassabehji et al. 1992). In fact, *Pax3* is intimately connected with *Mitf* and *Sox10* in other ways. *Pax3* binding sites have been identified in mouse and human *MITF* promoters and in vitro and in vivo activation of expression from these promoters by *PAX3* has been demonstrated (Bondurand et al. 2000, Lee et al. 2000, Potterf et al. 2000, Verastegui et al. 2000, Watanabe et al. 1998). Work in mouse and human cell culture studies have also found that *SOX10* and *PAX3* act synergistically to activate expression from the *MITF* promoter (Bondurand et al.

2000, Potterf et al. 2000) although others have found that in human cells, activation by SOX10 is not affected by co-expression of PAX3 nor by the loss of the PAX3 binding site (Lee et al. 2000, Verastegui et al. 2000). However, given that *mitfa* expression is lost entirely from *sox10* mutant zebrafish Pax3 is unlikely to be sufficient to promote *mitfa* expression in zebrafish.

The role of Pax3 in adult mouse melanocyte stem cell differentiation has been described in a similar network to the one we are exploring for Sox10 in zebrafish melanophores (Lang et al. 2005). In adult mouse melanocyte stem cells, Pax3 functions both to promote and to inhibit the differentiation of melanocyte stem cells. The authors identified Pax3 positive neural crest-derived cells in adult mouse hair follicles. Using an *Mitf* promoter fragment attached to a reporter they showed that at least in vitro Pax3 can activate expression of *Mitf*. In similar experiments they showed that Sox10 and *Mitf* are able to induce melanocyte differentiation by synergistically activating the *Dct* promoter. Under the same conditions that Pax3 is able to activate *Mitf*, Pax3 attenuates Sox10/*Mitf* induced *Dct* reporter expression and inhibits melanocyte differentiation. In fact, their work suggested that Pax3 and *Mitf* actually compete to bind the *Dct* promoter. Pax3 inhibition is relieved when it is displaced by activated  $\beta$ -catenin which then allows the melanocytes to fully differentiate. These findings support a general model for development whereby a single factor can both promote and inhibit differentiation to maintain cells in a state where they are poised to differentiate as Sox10 appears to do in our model. It is also plausible that Pax3 and Sox10 could both function in a more complex network in zebrafish to regulate melanophore development together.

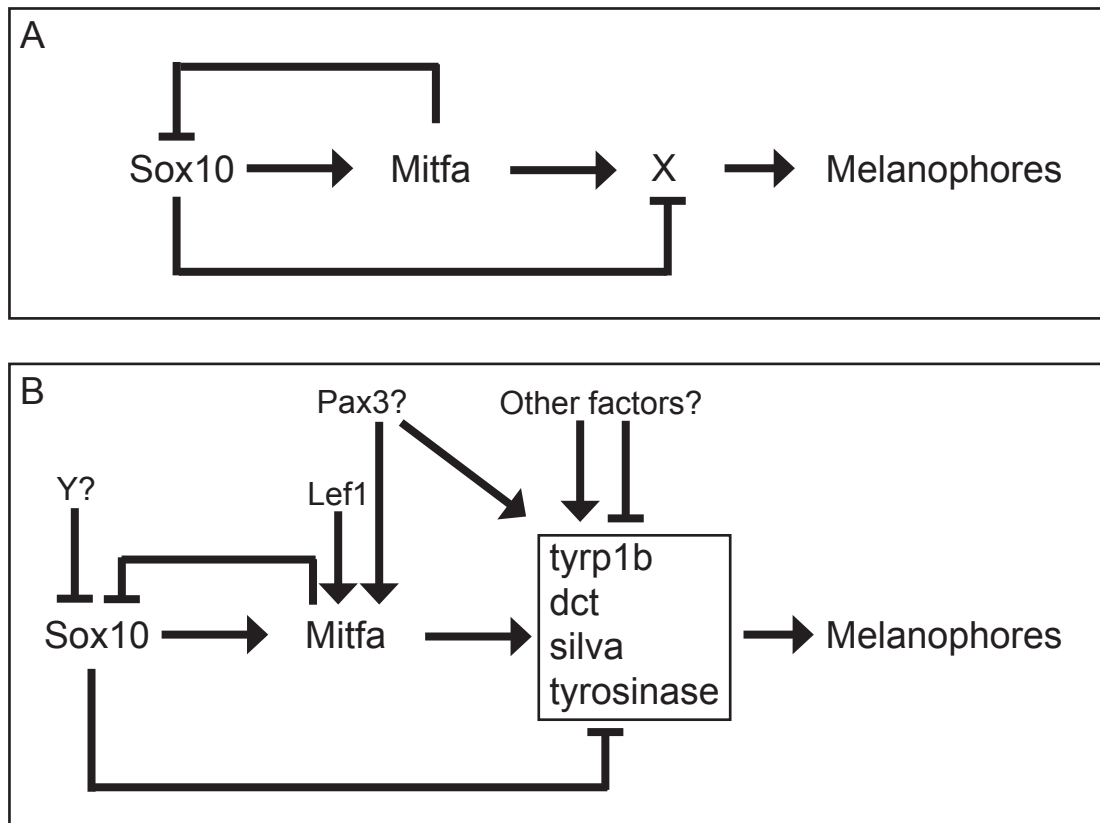
Study of Pax3 in relation to melanophore development in zebrafish is complicated slightly, both by the fact that zebrafish have two *Pax3* orthologues, *pax3* and *pax3b*, and by the fact that they have multiple pigment cell types. *pax3* and *pax3b* are both expressed in the premigratory neural crest but not in migrating cells (Minchin and Hughes 2008). Further information about *pax3b* is not available but morpholino knockdown of *pax3* causes a reduction in xanthophore number. There is also an early decrease in migrating cells which express melanophore markers, indicating a delay in melanophore migration which could disguise a potential delay in melanophore specification too. Later there is an increase in melanophore number which corresponds in severity to that of the reduction in xanthophore number (Minchin and Hughes 2008). The authors suggest that their results would be consistent with the presence of a chromatoblast in which Pax3 drives xanthophore fate specification whilst inhibiting melanophore specification (as it does in the mouse) so that in the absence of Pax3, xanthophores are reduced in number and melanophores are increased in number. The obvious importance of Pax3 in xanthophore specification is demonstrated in this paper,

together with evidence pointing towards the likely existence of chromatoblasts. The work also hints at a potential role for Pax3 in melanophores. It will be interesting to discover where this work leads. Might Pax3 in zebrafish both promote and inhibit melanophore development as it does in mouse? This would certainly not be incompatible with our model for the role of Sox10 in these cells. To learn more about how Pax3 might fit into our model it would be important to find out how whether Pax3 is required along with Sox10 for *mitfa* induction and whether it can directly affect the expression of our melanophore markers, perhaps by using an approach involving RNA injection experiments. Alternatively, the melanophore phenotype in *pax3* morphants might be an indirect effect of a primary role in xanthophores which results in the loss of potential for cells to become xanthophores in the absence of Pax3. It would then become particularly interesting to learn how Pax3b functions in the neural crest, since it could be that the role of zebrafish *pax3b* is more specific to melanocytes and more equivalent to the role of *Pax3* in mouse melanocytes.

### 6.3 Summary – A New Testable Model

We sought to explore the role of Sox10 in melanophore development by testing a model adapted from one for the role of Sox10 in mouse sympathetic neurons (Kim et al. 2003). We have succeeded in testing many aspects of this model. We knew previously that Sox10 activates expression of *mitfa* (Elworthy et al. 2003). Our data fully supports the conclusions drawn from Elworthy et al. (2003) that, in contrast to mouse, this is the only necessary role that Sox10 plays in melanophores. Whilst Sox10 does not appear to be required for the expression of any melanophore differentiation genes it does have a second role in melanophores. We have provided evidence in support of the hypothesis that Sox10 additionally inhibits melanophore development by inhibiting expression of genes downstream of Mitfa. Although further investigation is required, it appears that high levels of Sox10 may be required for this to occur, thus, Sox10 can still be present in pigmented melanophores. We have been able to document accurately that Sox10 and *sox10* are downregulated in melanophores over time. We were unable to test whether this downregulation is necessary for melanophores to differentiate but have found no evidence to the contrary. We have also generated some interesting data which shows that Mitfa can regulate expression of *sox10*. Our model predicts that this should be a negative relationship and whilst our experiments suggest the opposite is true, we believe that this possibility still remains open.

Our work has provided supporting evidence and answers for many of our hypotheses and questions but has also opened up new avenues for enquiry. We propose a new testable model (Figure 6.01) to describe the role of Sox10 in melanophores. The new



**Figure 6.01 A new testable model**

We have demonstrated that many of the predictions from our original model (A) hold true. However, we have also found that that model was an over simplification of the genetic network involved in melanophore differentiation. We suggest here some modifications to that model which make a new testable model that may mimic the in vivo network more closely (B, see text for details).

model is simplified in that there is no intermediate transcription factor X between *Mitfa* and the melanophore marker genes *dct*, *silva*, *tyrosinase* and *tyrp1b*. Although the concept of an intermediate transcription factor remains plausible, and we keep an open mind, there is currently no necessity for this extra factor and so we discard it from our model. In addition, we have provided evidence to suggest that activation of our melanophore specific genes by *Mitfa* is rapid and therefore likely to be direct. Overall, however, our new model actually depicts a more complex genetic network than our original model due to the inclusion of a number of different external factors. From our studies of the expression patterns of our marker genes and from our RNA injection experiments we concluded that *Sox10* and *Mitfa* were not the only factors that influenced the expression of our melanophore marker genes. Some of these other, unidentified, factors will have positive regulatory roles and others may have negative regulatory roles. We believe that the balance of which factors are most important, including *Sox10* and *Mitfa*, will vary between the different markers (Figure 3.17). We have suggested, from work in mouse and zebrafish, that *Pax3* may be one transcription factor involved but further evidence will need to be gathered in zebrafish to corroborate this suggestion. Detailed analysis of the promoters of our melanophore markers will reveal potential transcription factor binding sites and subsequent analysis of these will reveal which are the most relevant *in vivo* and so which transcription factors can be added to our new model. We also include a number of other factors in our new model which regulate the expression of *Sox10* and *Mitfa*. We know that *Sox10* is downregulated in differentiating melanophores and that *Mitfa* continues to be expressed. However, recent preliminary work using mathematical modelling to examine our original model has shown that a simple feedback loop between *Sox10* and *Mitfa* would never resolve itself so that *Sox10* expression is lost and *Mitfa* expression remains (Andrea Rocco, personal communication). Thus, unless a short burst of *Sox10* expression is enough to ensure persistent *mitfa* expression, it would be necessary for some other factors to also promote *mitfa* expression. Rocco also tested a mathematical model where a positive feedback loop is set up between *Mitfa* and another transcription factor, *Lef1* for example. In such a model, *Sox10* induces expression of *Mitfa* which in turn induces expression of *Lef1*; *Mitfa* and *Lef1* then reciprocally promote each others expression so that *Mitfa* can repress expression of *Sox10* without subsequent loss of its own expression. We can use the results from mathematical modelling to define the important parameters and characteristics of our regulatory network and direct further *in vivo* experiments. The results of these experiments can then feed back into the mathematical modelling allowing us to refine our model for the genetic regulatory network involved in zebrafish melanophore development.

We have tested our original model and provided evidence to support many of its

predictions. As is often the case in science however, this new knowledge poses many more questions than it answers and has provided many new lines of enquiry. Sox10 does not perform a single role in melanophores and it does not function in a simple genetic network either. Instead, our data suggests that it performs a number of roles in this cell type and that the genetic network surrounding it is more complex than we had first imagined. Further studies will need to take a much broader perspective when looking at the role of Sox10 and will probably find it to be at the centre of an ever expanding regulatory network.



# Appendices

## Appendix A – Reagents

### Oligos

BS1	AAC AAA AGC TGG AGC TCC ACC G
CFP-F	GCA GAA GAA CGG CAT CAA
CFP-R	GGT GCT CAG GTA GTG GTT GT
Linker 1	CGG GCG CCC TCG AGT CCG GAA TCG ATA CCG GTG TCG ACG GCC
Linker 2	GTC GAC ACC GGT ATC GAT TCC GGA CTC GAG GGC GCC
S19	GCA GCA AGA GCA AAC CGC ACG
S21	ACC TAC CGA AGT CAC CTG TGG
S22	GAT ATT GAT CCG CCA GTT TCC
SeqCFP	GGT CTT GTA GTT GCC GTC GT
SPACER1	CTG GAT ATC GAT TGA GGC
SPACER2	GCC TCA ATC GAT ATC
Spacerprimer	GAG GGC CTG GAT ATC GAT TGA
TYR1	AAG GCT CTT GGA TAC GAG TAC GCC

### Annealing Buffer

5 ml Tris HCl pH 7.5, 10 ml NaCl, 1 ml 0.5 M EDTA, 34 ml DEPC treated H<sub>2</sub>O

### Hybridisation Mix (for 50ml)

Formamide	25 ml
20x SSC	12.5 ml
Heparine (5 mg/ml)	0.5 ml
tRNA (50 mg/ml)	0.5 ml
Tween20 (20 %)	0.25 ml
Citric acid (1 M)	0.46 ml
Sterile water	10.7 ml

### NBT-BCIP Buffer

0.1 M Tris-HCl pH 9.5 at 20 °C, 0.1 M NaCl

### Embryo Medium (50x stock)

250 mM NaCl, 8.5 mM KCl, 16.5 mM CaCl<sub>2</sub>, 16.5 mM MgSO<sub>4</sub>

For 1x stock make up 200 ml 50x stock and 1 ml methylene blue in 10 L

### LacZ Staining Solution

In 500 ml PBS pH 7.4-7.5: 2 mM MgCl<sub>2</sub>, 0.01 % Sodium deoxycholate, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 0.02 % Nonidet P-40

### Genomic DNA Extraction Buffer

10 mM Tris pH 8.2, 10 mM EDTA, 200 mM NaCl, 0.5 % (v/v) SDS, 200 µg/ml Proteinase K

### Embryo Genomic DNA Extraction Buffer

10 mM Tris pH 7.5, 10 mM EDTA, 200 µg/ml Proteinase K

### Restriction Enzymes and Buffers Used

#### NEB Buffers

Buffer 1: 10 mM Bis-Tris-Propane-HCl, 10 mM MgCl<sub>2</sub>, 1 mM Dithiothreitol, pH 7.0 at 25 °C

Buffer 2: 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM Dithiothreitol, pH 7.9 at 25 °C

Buffer 3: 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM Dithiothreitol, pH 7.9 at 25 °C

Buffer 4: 50 mM Potassium Acetate, 20 mM Tris-Acetate, 10 mM Magnesium Acetate, 1 mM Dithiothreitol, pH 7.9 at 25 °C

#### Promega Buffers

Buffer A: 6 mM Tris-HCl, 6 mM MgCl<sub>2</sub>, 6 mM NaCl, 1 mM Dithiothreitol, pH 7.5 at 37 °C

Buffer B: 6 mM Tris-HCl, 6 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mM Dithiothreitol, pH 7.5 at 37 °C

Buffer C: 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mM Dithiothreitol, pH 7.9 at 37 °C

Buffer D: 6 mM Tris-HCl, 6 mM MgCl<sub>2</sub>, 150 mM NaCl, 1 mM DTT, pH 7.9 at 37 °C

Enzyme Name	Buffer	Manufacturer
AgeI	1	NEB
ApaI	A	Promega
BspEI	3	NEB
ClaI	C	Promega
EcoRV	D	Promega
KasI	2	NEB
KpnI	J	Promega
MluI	3	NEB
MspI	B	Promega
NdeI	4	NEB
Sall	D	Promega
SfoI	2	NEB
XhoI	D	Promega

## Appendix B – Residual Melanophore Counts in *sox10*<sup>t3</sup> Mutant Embryos

Embryos were tracked individually at different time points. We scored each embryo for presence or absence of melanised cells in each somite. The data from each embryo are represented here in tables. Each table corresponds to one embryo, blue coloured boxes indicate that there was at least one melanised cell in the corresponding somite, a blank box indicates that there was not. Embryos 1-19, 20-34 and 25-29 were scored on three separate occasions.

[illegible]

E2	Hpf	Somite 1
	S2	
	S3	
	S4	
	S5	
	S6	
	S7	
	S8	
	S9	
	S10	
	S11	
	S12	
	S13	
	S14	
	S15	
	S16	
	S17	
	S18	
	S19	
	S20	
	S21	
	S22	
	S23	
	S24	

[illegible][illegible][illegible]

E6	Somite 1
Hpf	S2
	S3
	S4
	S5
	S6
	S7
	S8
	S9
	S10
	S11
	S12
	S13
	S14
	S15
	S16
	S17
	S18
	S19
	S20
	S21
	S22
	S23
	S24

E7	Somite 1																							
Hpf	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	
36																								
40																								
66																								

E8	Somite 1																							
Hpf	Somite 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
36																								
40																								
66																								

E9	Somite 1																							
	Hpf	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
36																								
40																								
66																								

E10	Somite 1																							
Hpf	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	
36																								
40																								
66																								

E11	Somite 1																							
Hpf	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	
36																								
40																								
66																								

E12	Somite 1																							
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E13	Somite 1																							
Hpf	Somite 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
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E20	Hp	Somite 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
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E21	Hp	Somite 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
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E22	Hp	Somite 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
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E23	Hp	Somite 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
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E24	Hp	Somite 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
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E25	Somite 1																							
Hpf	Somite 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
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E26	Somite 1																							
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E27	Somite 1																							
Hpf	Somite 1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24
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E28	Somite 1																							
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E29	Somite 1																							
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August 22, 2008

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